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Application No. 09/513,086	Filing Date 02/24/2000	Examiner Joseph T. Woitach	Customer No. 21036	Group Art Unit 1632

Invention: VACCINE TO CONTROL EQUINE PROTOZOAL MYELOENCEPHALITIS IN HORSES

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Appl. No. : 09/513,086 Confirmation No. 4724

Applicants : Linda S. Mansfield, Mary G. Rossano, Alice J. Murphy, and Ruth A. Vrable

Filed : February 24, 2000

Title: VACCINE TO CONTROL EQUINE PROTOZOAL MYELOENCEPHALITIS IN HORSES

TC/A.U. : 1632

Examiner : Woitach, Joseph T.

Docket No. : MSU 4.1-458

Customer No. : 21036

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SUPPLEMENTAL BRIEF UNDER 37 C.F.R. § 41.37

Sir:

This is in response to the Notice of Non-Complaint Appeal Brief dated June 06, 2006. This is an appeal from a final rejection in the above entitled application. The claims on appeal are set forth as Claims Appendix. An oral hearing will be requested. The fee due upon filing of the Brief has been paid.

(1) Real Party in Interest

The real party in interest is the Board of Trustees operating Michigan State University, East Lansing, Michigan, a constitutional corporation of the State of Michigan, which is the assignee of the above entitled application.

(2) Related Appeals and Interferences

The present application is Application Serial No. 09/513,086 ('086), filed February 24, 2000, and which claims benefit of a provisional patent Application No. 60/152,193, filed September 2, 1999.

This application is related to Application Serial No. 09/669,833 ('833) which relates to a method for producing an antibody for use as a passive immunity vaccine in horses against a *Sarcocystis neurona* antigen selected from the group consisting of a 16 (+/-4) kDa antigen and a 30 (+/-4) kDa antigen; Application Serial No. 09/669,843 ('843) which relates to a monoclonal antibody which selectively binds to a *Sarcocystis neurona* antigen; Application Serial No. 09/670,096 ('096), relating to compositions and method for treating an equid infected with

Sarcocystis neurona with antibodies against the 16 ±4 and 30 ±4 kDa antigens; Application Serial No. 09/670,244 ('224) which relates to recombinant protein comprising the 16 ±4 and 30 ±4 kDa antigens; and Application Serial No. 09/670,355 ('355), relating to a vaccine comprising DNA encoding the 16 ±4 and 30 ±4 kDa antigens. The above applications were all filed on September 26, 2000.

The '355 application was abandoned after an affirmation by the Board. The '244 application has been abandoned. A decision by the Board is enclosed for Application Serial No. 09/670,096 ('096) and for Application Serial No. 09/669,843 ('843). Application Serial No. 09/669,833 ('833) is on appeal. No application has been allowed. There are no other related appeals and interferences.

(3) Status of Claims

Claims 4, 13, 46 and 50 are pending in the application. Claims 1-3, 5-12, 14-45, 47-49 were cancelled. Claims 4, 13, 46 and 50 were rejected. No claims have been allowed. Claims 4, 13, 46 and 50 are on appeal.

(4) Status of Amendments

No amendments have been filed subsequent to final rejection.

(5) Summary of Claimed Subject Matter

The claimed subject matter in Claim 4 is a composition consisting of a single naturally occurring 16 (±4) kDa protein antigen isolated from *Sarcocystis neurona* and a single naturally occurring 30 (±4) kDa protein antigen isolated from *Sarcocystis neurona* in a pharmaceutically accepted carrier. Support for this claim is found in Example 1, page 33, lines 25-34.

The claimed subject matter in Claim 13 is a method for treating an equine with a *Sarcocystis neurona* infection comprising: (a) providing a composition consisting of a single naturally occurring 16 (±4) kDa protein antigen isolated from *Sarcocystis neurona* and a single naturally occurring 30 (±4) kDa protein antigen isolated from *Sarcocystis neurona* in a pharmaceutically accepted carrier; and (b) inoculating the equine with the composition to treat the equine with the *Sarcocystis neurona* infection. Support

for this claim is found in Example 1, page 33, lines 25-34. Support for using the vaccine as a vaccine for an equine is found at page 13, lines 1-5. Support for inoculating the equine is found at page 14, lines 32 to page 15, line 25.

The claimed subject matter in Claim 46 is a method for treating a disease in an equine caused by a *Sarcocystis neurona* infection which comprises providing a composition which when injected into the equine causes the equine to produce antibodies against a 16 (±4) kDa antigen and a 30 (±4) kDa antigen of the *Sarcocystis neurona* which treats the disease caused by the *Sarcocystis neurona,* wherein the composition consists of a single naturally occurring 16 (±4) kDa protein antigen isolated from *Sarcocystis neurona* and a single naturally occurring 30 (±4) kDa protein antigen isolated from *Sarcocystis neurona* in a pharmaceutically accepted carrier. Support for this claim is found in Example 1, page 33, lines 25-34. Support for using the vaccine as a vaccine for an equine is found at page 13, lines 1-5. Support for inoculating the equine is found at page 14, lines 32 to page 15, line 25. Support for treatment is found at page 10, lines 22-34. Support for causing the equine to produce antibodies is found at page 9,

lines 22-31.

The claimed subject matter in Claim 50 is a method of Claim 46 wherein the composition is administered by an inoculation route selected from the group consisting of intranasal administration, intramuscular injection, intraperitoneal injection, intradermal injection, and subcutaneous injection. Support for this claim is found in Example 1, page 33, lines 25-34. Support for using the vaccine as a vaccine for an equine is found at page 13, lines 1-5. Support for inoculating the equine is found at page 14, lines 32 to page 15, line 25. Support for the route of administration is found at page 13, lines 24-35.

(6) Grounds of Rejection to Be Reviewed on Appeal

(A) The Examiner rejected Claims 4, 13 and 46 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

(B) The Examiner rejected Claims 4, 13 and 46 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, since a disclosure cannot teach one to make or use something that has not been described.

(C) Claims 4, 13, 45, 46 and 50 were rejected under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

(7) Argument

(A) The Examiner rejected Claims 4, 13 and 46 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

The subject matter of a claim need not be described literally (*i.e.*, using the same terms or *in haec verba*) in order for the disclosure to satisfy the description requirement (MPEP §2163.02). While the term "naturally occurring" is not found in the text, according to Example 1 on page 33 of the specification:

> "*Sarcocystis neurona* was cultured on equine dermal cell line cultures as taught in Example 3 or on bovine monocyte cell cultures as taught by Granstrom *et al.*, *J. Vet. Diagn. Invest.* 5: 88-90 (1993). *Sarcocystis neurona* merozoites were harvested and the 16 (±4) kDa antigen and/or 30 (±4) kDa antigen were purified by methods known to the art for purifying antigens, i.e., the 16 (±4) kDa antigen and/or 30 (±4) kDa antigen were purified from merozoites by two-dimensional polyacrylamide gel electrophoresis. Then the purified antigens are used to make monoclonal antibodies according to the methods in *Antibodies, A Laboratory Manual*, eds. Harlow and Lane, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988),

well known to those skilled in the art as a source for methods for making polyclonal and monoclonal antibodies."

According to MPEP §2163.02, whenever the issue arises, the fundamental factual inquiry is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed. *See, e.g., Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991). An applicant shows possession of the claimed invention by describing the claimed invention with all of its limitations using such descriptive means as words, structures, figures, diagrams, and formulas that fully set forth the claimed invention. *Lockwood v. American Airlines, Inc.*, 107 F.3d 1565, 1572, 41 USPQ2d 1961, 1966 (Fed. Cir. 1997). Possession may be shown in a variety of ways including description of an actual reduction to practice, or by showing that the invention was "ready for patenting" such as by the disclosure of drawings or structural chemical formulas that show that the invention was complete, or by describing distinguishing identifying characteristics sufficient to show that the applicant was in possession of the claimed invention. *See, e.g., Pfaff v.*

Wells Elecs., Inc., 525 U.S. 55, 68, 119 S.Ct. 304, 312, 48 USPQ2d 1641, 1647 (1998); *Regents of the University of California v. Eli Lilly*, 119 F.3d 1559, 1568, 43 USPQ2d 1398, 1406 (Fed. Cir. 1997); *Amgen, Inc. v. Chugai Pharmaceutical*, 927 F.2d 1200, 1206, 18 USPQ2d 1016, 1021 (Fed. Cir. 1991) (one must define a compound by "whatever characteristics sufficiently distinguish it"). As seen in Example 1, the 16 (±4) kDa antigen and 30 (±4) kDa antigen are purified from *Sarcocystis neurona* cultures and are thus naturally occurring proteins isolated from *Sarcocystis neurona*. In this example, mice are injected with the purified 16 (±4) kDa antigen and 30 (±4) kDa antigen to produce antibodies. Thus, the purified proteins are used as antigens for the production of antibodies. Therefore, the claimed subject matter was described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Reversal of the rejection is requested.

(B) The Examiner rejected Claims 4, 13 and 46 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, since a disclosure cannot teach one to make or use something that has not been described.

There are many factors to be considered when determining whether there is sufficient evidence to support a determination that a disclosure does not satisfy the enablement requirement and whether any necessary experimentation is "undue." These factors include, but are not limited to:

(A) The breadth of the claims;
(B) The nature of the invention;
(C) The state of the prior art;
(D) The level of one of ordinary skill;
(E) The level of predictability in the art;
(F) The amount of direction provided by the inventor;
(G) The existence of working examples; and
(H) The quantity of experimentation needed to make or use the invention based on the content of the disclosure.

In re Wands, 858 F.2d 731, 737, 8 USPQ2d 1400, 1404 (Fed.

Cir. 1988). The examiner's analysis must consider all the evidence related to each of these factors, and any conclusion of nonenablement must be based on the evidence as a whole. 858 F.2d at 737, 740, 8 USPQ2d at 1404, 1407.

Considering the state of the prior art of protein isolation, the direction provided by the inventors in Example 1, and the high level of one or ordinary skill in the art the subject matter was described in the specification in such a way as to enable one skilled in the art to make the invention. The quantity of experimentation needed to be performed by one skilled in the art is only one factor involved in determining whether "undue experimentation" is required to make and use the invention. "[A]n extended period of experimentation may not be undue if the skilled artisan is given sufficient direction or guidance." *In re Colianni*, 561 F.2d 220, 224, 195 USPQ 150, 153 (CCPA 1977). "The test is not merely quantitative, since a considerable amount of experimentation is permissible, if it is merely routine, or if the specification in question provides a reasonable amount of guidance with respect to the direction in which the experimentation should proceed." *In re Wands*, 858 F.2d 731,

737, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988) (citing In re Angstadt, 537 F.2d 489, 502-04, 190 USPQ 214, 217-19 (CCPA 1976)). It is believed that the example provides sufficient information such that a person of ordinary skill in the art can make and/or use the invention.

M.P.E.P. 2164.01(b) states, that as long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement of 35 U.S.C. 112 is satisfied. *In re Fisher*, 427 F.2d 833, 839, 166 USPQ 18, 24 (CCPA 1970). The 16 (±4) kDa antigen and 30 (±4) kDa antigen are each purified from *Sarcocystis neurona* cultures as described in Example 1. The specification specifically teaches in Example 1 that the 16 (±4) and 30 (±4) kDa antigens are isolated by two-dimensional gel electrophoresis (page 33, lines 29-34). These are therefore naturally occurring proteins which are isolated from *Sarcocystis neurona*. In addition, it is taught in Example 1 that mice can be injected with the purified 16 (±4) kDa antigen and 30 (±4) kDa antigen to produce antibodies. Therefore the specification enables one skilled in the art to make and/or

use the invention. Reversal of the rejection is requested.

(C) The Examiner rejected Claims 4, 13, 46 and 50 under 35 U.S.C. §112, first paragraph, as containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention.

An objective standard for determining compliance with the written description requirement is, "does the description clearly allow persons of ordinary skill in the art to recognize that he or she invented what is claimed." *In re Gosteli*, 872 F.2d 1008, 1012, 10 USPQ2d 1614, 1618 (Fed. Cir. 1989). Under *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991), to satisfy the written description requirement, an applicant must convey with reasonable clarity to those skilled in the art that, as of the filing date sought, he or she was in possession of the invention, and that the invention, in that context, is whatever is now claimed. The test for sufficiency of support in a parent application is whether

the disclosure of the application relied upon "reasonably conveys to the artisan that the inventor had possession at that time of the later claimed subject matter." *Ralston Purina Co. v. Far-Mar-Co., Inc.*, 772 F.2d 1570, 1575, 227 USPQ 177, 179 (Fed. Cir. 1985) (quoting *In re Kaslow*, 707 F.2d 1366, 1375, 217 USPQ 1089, 1096 (Fed. Cir. 1983)). Whenever the issue arises, the fundamental factual inquiry is whether the specification conveys with reasonable clarity to those skilled in the art that, as of the filing date sought, applicant was in possession of the invention as now claimed. See, e.g., *Vas-Cath, Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 USPQ2d 1111, 1117 (Fed. Cir. 1991).

The claimed composition consists of a single naturally occurring 16 (±4) kDa protein antigen isolated from *Sarcocystis neurona* and a single naturally occurring 30 (±4) kDa protein antigen isolated from *Sarcocystis neurona* in a pharmaceutically accepted carrier. The claimed methods utilize this composition to treat the equine with the *Sarcocystis neurona* infection. The composition consists of naturally occurring protein antigens which are isolated from *Sarcocystis neurona*, and is not directed to polypeptide fragments, recombinant polypeptides or fusion polypeptides.

As the claims are specifically directed to the isolated form of the naturally occurring proteins, it is believed that the written description requirement of 35 U.S.C. §112, first paragraph is satisfied.

The 16 (±4) and 30 (±4) kDa antigens are described in the specification by their physical properties, not merely by their function. The 16 (±4) and 30 (±4) kDa antigens are described by their source (isolated from *Sarcocystis neurona*), by their molecular weight as determined by SDS gel electrophoresis, by their ability to bind particular antibodies in antisera from horses infected with *Sarcocystis neurona*, and by their ability to bind monoclonal antibodies prepared against them. These physical properties convey sufficient information about the antigens to distinguish them from the other proteins of *Sarcocystis neurona*. There is no need to know the amino acid sequence of the antigens or the nucleotide sequence encoding the antigens to identify them. The specification describes the 16 (±4) and 30 (±4) kDa antigens by their respective mobilities on SDS polyacrylamide gels (Page 36, lines 22-27 of the specification; U.S. Serial No. 09/156,954, which is now U.S. Patent No. 6,153,394 to Mansfield *et al.*,

incorporated by reference on page 13, lines 16-17 of the specification) and two-dimensional gels (Specification: page 33, lines 29-34), by their ability to bind antibodies in antisera from horses infected with *Sarcocystis neurona* (U.S. Patent No. 6,153,394), and by their inability to bind antibodies from other *Sarcocystis* species (Page 13, lines 20-21 of the specification).

The specification further teaches in Example 1 that the 16 (±4) and 30 (±4) kDa antigens were isolated by two-dimensional gel electrophoresis (page 33, lines 29-34) and teaches a method for preparing monoclonal antibodies using the purified 16 (±4) and 30 (±4) kDa antigens. The monoclonal antibodies can be used to identify the 16 (±4) and 30 (±4) kDa antigens (Example 1 at page 33, line 20 of the specification). Therefore, a person of ordinary skill in the art following the teachings in the specification of the present application would be able to identify and isolate the 16 (±4) and 30 (±4) kDa antigens of *Sarcocystis neurona*. Furthermore, the applicants are not claiming the 16 (±4) and 30 (±4) kDa antigens *per se*. They are claiming a composition that consists of naturally occurring protein antigens, which can be isolated by described methods from a

known source. The written description is believed to be adequate without the necessity of providing the amino acid sequence of the proteins comprising the composition. Reversal of the rejection is requested.

B. Conclusion

As shown above, the claimed subject matter is described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors had possession of the claimed subject matter. Also, the claimed subject matter was described in the specification in such a way as to enable one skilled in the art to which it pertains to make and/or use the invention. Therefore, Claims 4, 13, 46 and 50 are patentable. Reversal of the Final Rejection is requested.

Respectfully,

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CLAIMS APPENDIX

4. A composition consisting of a single naturally occurring 16 (±4) kDa protein antigen isolated from *Sarcocystis neurona* and a single naturally occurring 30 (±4) kDa protein antigen isolated from *Sarcocystis neurona* in a pharmaceutically accepted carrier.

13. A method for treating an equine with a *Sarcocystis neurona* infection comprising:

(a) providing a composition consisting of a single naturally occurring 16 (±4) kDa protein antigen isolated from *Sarcocystis neurona* and a single naturally occurring 30 (±4) kDa protein antigen isolated from *Sarcocystis neurona* in a pharmaceutically accepted carrier; and

(b) inoculating the equine with the composition to treat the equine with the *Sarcocystis neurona* infection.

46. A method for treating a disease in an equine caused by a *Sarcocystis neurona* infection which comprises providing a composition which when injected into the equine causes the equine to produce antibodies against a 16 (±4) kDa antigen and a 30 (±4) kDa antigen of the *Sarcocystis neurona* which treats the disease caused by the *Sarcocystis neurona,* wherein the composition consists of a single naturally occurring 16 (±4) kDa protein antigen isolated from *Sarcocystis neurona* and a single naturally occurring 30 (±4) kDa protein antigen isolated from *Sarcocystis neurona* in a pharmaceutically accepted carrier.

50. The method of Claim 46 wherein the composition is administered by an inoculation route selected from the group consisting of intranasal administration, intramuscular injection, intraperitoneal injection, intradermal injection, and subcutaneous injection.

EVIDENCE APPENDIX

Attached are copies of cited patents and publications:

1. Granstrom *et al.*, *J. Vet. Diagn. Invest.* 5: 88-90 (1993). Submitted in Information Disclosure Statement as citation E. Considered by Examiner Yvette Connell on 7/17/00.

2. *Antibodies, A Laboratory Manual*, eds. Harlow and Lane, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York (1988). Submitted in Information Disclosure Statement as citation N. Considered by Examiner Yvette Connell on 7/17/00.

3. U.S. Patent No. 6,153,394 to Mansfield *et al.* (Application No. 09/156,954).

RELATED PROCEEDINGS APPENDIX

Attached are copies of the following:

1. Decision by the Board for Application No. 09/670,355 ('355).

2. Decision by the Board for Application No. 09/670,096 ('096).

3. Decision by the Board for Application No. 09/669,843 ('843).

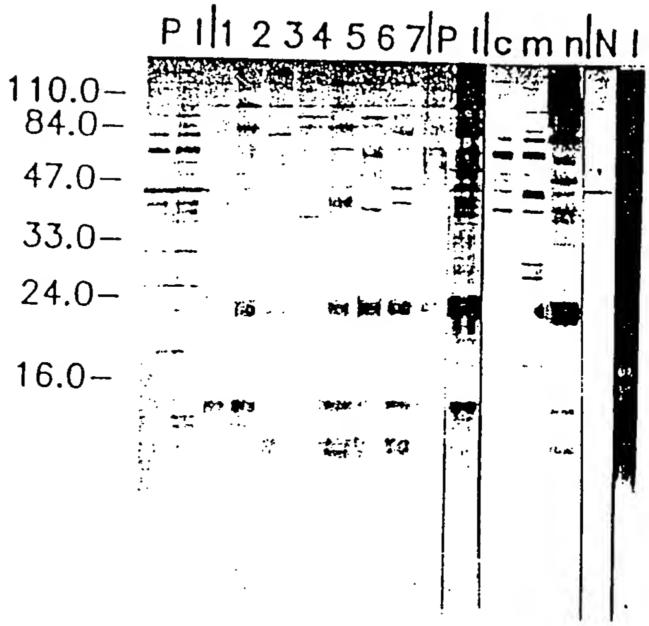

Figure 2. Immunoblot of solubilized *S. neurona* merozoites separated on a 10–20% linear gradient SDS-PAGE gel. The blot was probed with the following sera: pre- (P) and post-infection (I) *S. fayeri* (Sf) sera from a pony, sera from 7 histologically confirmed cases of equine protozoal myeloencephalitis (EPM 1–7), pre- (P) and post-inoculation (I) *S. neurona* (Sn) sera from a horse, rabbit anti-*S. cruzi* (c), *S. muris* (m), and *S. neurona* (n) sera and normal rabbit serum (N). Prestained molecular weight markers (MW) and India ink stain (I) are indicated.

were *S. neurona*-specific. If the *S. fayeri*-infected pony or *S. neurona*-inoculated horse were exposed previously to *S. neurona*, additional specific proteins might have been undetected. As indicated in Tables 1 and 3, *S. neurona*-specific proteins, 22.5, 13, and 10.5 Kd, were recognized by all 7 horses with EPM and the *S. neurona*-inoculated horse. These proteins should be especially useful for antemortem detection of *S. neurona* exposure among clinical cases and to determine the seroprevalance of specific antibodies among groups of horses and other animals. They also are potential candidates for development of an *S. neurona*-specific immunoassay.

Immunoblot analysis of *S. neurona* merozoite antigens using the 3 rabbit antisera demonstrated many epitopes shared with *S. cruzi* and *S. muris* bradyzoites. Recognition of 6 of the 8 *S. neurona*-specific proteins by rabbit anti-*S. neurona* serum but not by rabbit anti-*S. muris* or *S. cruzi* sera supports the results obtained with equine sera.

Serum IFA titers did not provide clear differentiation between exposure to *S. fayeri* or *S. neurona*. *Sarcocystis fayeri* infection or *S. neurona* inoculation resulted in titers of only 50 and 25, respectively. Titers were not related to the number of specific proteins recognized on immunoblots. Two of the horses that recognized all 8 specific proteins had low IFA titers. The chronology of the disease, i.e., sporocyst dose, length of exposure, dissemination of merozites, and treatment regimen, among EPM-affected horses might have affected results. Some of the proteins recognized by sera from cultured merozoites might have been associated with late schizogonous generations that were present only during the chronic phase of natural infection. Paired or sequential serum samples were not available for evaluation.

Acknowledgements

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Sources and manufacturers

a. Percoll, Pharmacia, Uppsala, Sweden.
b. High- and low-range SDS-PAGE molecular weight markers, Bio-Rad Laboratories, Richmond, CA.
c. Low-range, prestained SDS-PAGE molecular weight markers, Bio-Rad Laboratories, Richmond, CA.
d. Antifoam-B, Sigma Chemical Co., St. Louis, MO.
e. Miniblotter 25, Immunetics, Cambridge, MA.
f. Pierce Chemical Co., Rockford, IL.
g. Zymed Laboratories, San Francisco, CA.
h. Sigma Chemical Co., St. Louis, MO.

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7. Granstrom DE, Ridley RK, Baoan Y: 1990, Immunodominant proteins of *Sarcocystis cruzi* bradyzoites isolated from cattle affected or nonaffected with eosinophilic myositis. Am J Vet Res 51:1151–1156.
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J Vet Diagn Invest 5:88-90 (1993)

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Equine protozoal myeloencephalitis: antigen analysis of cultured *Sarcocystis neurona* merozoites

David E. Granstrom, J. P. Dubey, Stan W. Davis, Ronald Fayer, J. Carl Fox, K. B. Poonacha, Ralph C. Giles, Patrick F. Comer

Abstract. Antigens of cultured *Sarcocystis neurona* merozoites were examined using immunoblot analysis. Blotted proteins were probed with *S. cruzi*, *S. muris*, and *S. neurona* antisera produced in rabbits. *S. fayeri* (pre- and post-infection) and *S. neurona* (pre- and post-inoculation) sera produced in horses, immune sera from 7 histologically confirmed cases of equine protozoal myeloencephalitis (EPM), and pre-suckle serum from a newborn foal. Eight proteins, 70, 24, 23.5, 22.5, 13, 11, 10.5, and 10 Kd, were detected only by *S. neurona* antiserum and/or immune serum from EPM-affected horses. Equine sera were titered by the indirect immunofluorescent antibody (IFA) method using air-dried, cultured *S. neurona* merozoites. Anti-*Sarcocystis* IFA titers were found in horses with or without EPM. Serum titers did not correspond to the number of specific bands recognized on immunoblots.

Equine protozoal myeloencephalitis (EPM) is an often debilitating central nervous system (CNS) disease of the horse.[9] It has been reported in horses native to North,[5] South,[1] and Central America.[6] The etiologic agent of EPM was recently cultured from naturally occurring cases in the US[2,3] and Panama[6] and named *Sarcocystis neurona*.

Serological diagnosis of EPM caused by *S. neurona* has been complicated by the fact that species within the genus *Sarcocystis* share antigens.[4] For this reason, cross-reactions are observed when antigen from one species is recognized by serum antibody developed against another species. The objective of this study was to identify *S. neurona*-specific proteins for antemortem diagnostic use that would not cross-react with antibody against other species.

Materials and methods

Sarcocystis neurona merozoites were harvested from bovine monocyte (M617) cell cultures and purified on an isosmotic colloidal silica[a] step gradient. One million purified merozoites or 5×10^4 M617 cells were solubilized in 0.125 M tris-Cl, pH 6.8 with 4% SDS, 20% glycerol, 10% 2-mercaptoethanol and boiled for 1 min. Solubilized merozoites and cells were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 0.75 mm, vertical, 5–20% or 10–20% linear gradient polyacrylamide resolving gels and 4% stacking gels using a discontinuous buffer method[8] and silver stained.[7] High- and low-range molecular weight markers were included in separate lanes.[b]

Immunoblots were prepared using 1×10^7 solubilized merozoites separated in 1-well SDS-PAGE gels. Separated proteins were electrophoretically transferred to 0.45 μm nitrocellulose in Towbin buffer (0.192 M glycine, 0.025 M tris pH 8.3, 20% v/v methanol) using 1.0 A constant current for 1 hr. Prestained molecular weight markers were used in a separate lane.[c] Blotting efficiency was demonstrated by India ink staining of the blot and Coomassie blue staining of the transferred gel.[7] Blots were blocked with 5% non-fat dry milk, 0.1% nonionic emulsifier,[d] and 0.1% sodium azide in 0.01 M PBS, pH 7.2 for 1 hr and subdivided into lanes using a molded plexiglass press.[e] Each of the following sera were diluted in blocking buffer and incubated for 2 hr in individual lanes: *S. cruzi* (bradyzoites), *S. muris* (bradyzoites), and *S. neurona* (merozoites) antisera prepared in rabbits,[7] normal rabbit serum, *S. fayeri* (pre- and post-infection) and *S. neurona* (pre- and post-inoculation with 3×10^8 merozoites 3 times at 2-wk intervals) antisera produced in horses, immune sera from 7 histologically confirmed cases of EPM, and pre-suckle serum from a newborn foal. Rabbit sera were diluted 1:100 and equine sera were diluted 1:10.

Antibodies were detected using biotinylated anti-equine IgG[f] or biotinylated anti-rabbit IgG[f] diluted 1:300 in PBS followed by streptavidin-peroxidase conjugate[f] (1:2,500) and aminoethyl carbazole/hydrogen peroxide chromogen/substrate. Blots were washed in 0.01 M PBS, pH 7.2 with 0.1% tween-20 4 times for 2 min between each incubation step.

Cultured merozoites were air-dried on methanol-cleaned microscope slides for IFA. Slides were incubated in 2-fold serial dilutions of serum beginning at 1:25 in 0.01 M PBS, pH 7.2, with 0.5% bovine serum albumin (BSA). Slides were

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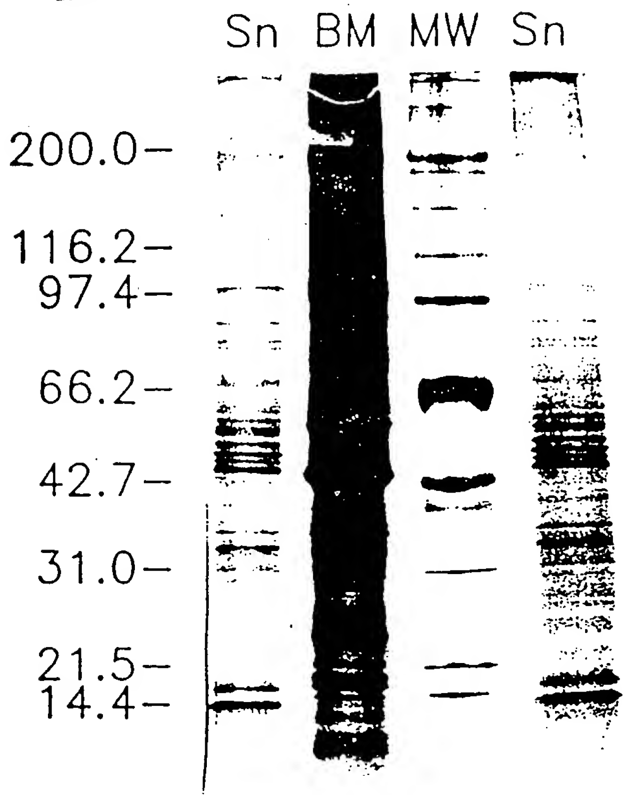

Figure 1. Silver stained 5–20% linear gradient SDS-PAGE gel of solubilized *S. neurona* merozoites (Sn), bovine monocytes (BM), and molecular weight markers (MW).

washed in PBS-BSA and incubated in 1:250 rabbit anti-equine IgG-fluoroscein isothiocyanate conjugate[h] in PBS-BSA. Titers were reported as the reciprocal of the highest serum dilution that produced fluorescing merozoites.

Results

Silver staining detected 104 proteins ranging in size from 14 to 300 Kd (Fig. 1). Although many proteins were recognized on immunoblots by most sera, 8 proteins were detected by sera from EPM-affected or *S. neurona*-inoculated horses that were not recognized by pre- or post-infection sera from the *S. fayeri*-infected pony or pre-inoculation serum from the *S. neurona*-inoculated horse (Table 1). Six of these also were recognized by rabbit anti-*S. neurona* serum but not by rabbit anti-*S. cruzi* or *S. muris* sera (Table 1). Presuckle foal serum detected no proteins (data not shown). Normal rabbit serum reacted with 3 bands common to many of the sera. India ink staining demonstrated successful transfer of proteins to the nitrocellulose.

Immunofluorescent antibody titers are listed in Table 1. Titers below 25 were considered negative based on previous tests of sera from horses without neurologic signs. Rabbit antisera were not tested.

Discussion

Immunoblot and IFA results indicated that the horses in this study previously were exposed to *Sarcocystis* sp. or other organisms containing cross-reactive epitopes. Immunoblot analysis detected 8 proteins that

Table 1. Molecular weights (Kd) of *Sarcocystis neurona*-specific proteins detected by immunoblot analysis. Sera used to develop immunoblots are listed across the top row of the table; molecular weights are listed in the far left column. *Sarcocystis neurona* IFA titers for each serum are indicated.

	Sf†		EPM‡							Sn§		S‖		
MW*	P	I	1	2	3	4	5	6	7	P	I	c	m	n
70	−¶	−	+#	−	+	+	+	+	+	−	+	−	−	−
24	−	−	+	−	−	−	−	+	+	−	+	−	−	+
23.5	−	−	+	+	−	−	+	+	+	−	+	−	−	+
22.5	−	−	+	+	+	+	+	+	+	−	+	−	−	+
13	−	−	+	+	+	+	+	+	+	−	+	−	−	+
11	−	−	+	−	+	−	+	+	+	−	−	−	−	−
10.5	−	−	+	+	+	±**	+	+	+	−	+	−	−	+
10	−	−	+	−	+	−	+	+	+	−	−	−	−	+
IFA	<25	50	100	25	100	50	400	50	25	<25	25	ND††	ND	ND

* MW = molecular weight of *S. neurona*-specific proteins, Kd.
† Sf, P, I = *S. fayeri* pre- (P) and post-infection (I) sera.
‡ EPM 1–7 = immune sera from 7 histologically confirmed EPM cases.
§ Sn, P, I = *S. neurona* pre- (P) and post-inoculation (I) sera.
‖ S, c, m, n = rabbit anti-*S. cruzi*, *S. muris*, and *S. neurona* sera.
¶ − = protein not recognized by serum.
# + = protein recognized by serum.
** ± = protein very lightly reactive with serum.
†† ND = not done.

Antibodies

A LABORATORY MANUAL

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CSH

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1988

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PREFACE

This manual was inspired by "the cloning manual," *Molecular Cloning: A Laboratory Manual* by Tom Maniatis, Ed Fritsch, and Joe Sambrook, a book that did so much to expand the community of scientists using molecular biology methods. The great joy of that manual was and is the confidence it gives to beginners and the way it tempts all workers to try new to approaches. At present, many of the methods of modern immunochemistry seem beyond the reach of nonimmunologists, and one of the goals of *Antibodies* is to make these methods accessible to a wider group. We hope that this manual is judged by how well it succeeds in breaking this barrier.

Antibodies has a long and complex genesis. The original ideas for an immunology manual were developed with Ron McKay, Steve Blose, and Jim Lin. Some portions of those early plans remain in this version, and we would like to thank Ron, Steve, and Jim for their contributions. Version 2, the eventual precursor to this book, was conceived in a naive burst of enthusiasm in the summer of 1986. In those early days, we talked of pooling our lab protocols and finishing quickly. As we worked and talked to our colleagues, two things became clear. First, there seemed to be a genuine need for a practical guide to immunochemical methods for the nonimmunologist (which was encouraging), and second, the gap between modern immunology and the backgrounds of most molecular biologists was larger than we had thought. This meant that more background information was needed if the book were to help a reasonable cross section of scientists (which was less encouraging).

The final version of *Antibodies* contains four introductory chapters that summarize the key features of the immune response, the structure of the antibody molecule, the activities of antibodies, and the mechanism of the antibody response. These summaries are designed to be an up-to-date consensus, and they will no doubt excite some ire among immunologists by their oversimplifications and omissions, but we hope that the nonspecialist will find them a helpful framework for the ideas and techniques presented in later chapters. The bulk of the book contains protocols for raising, purifying, and labeling monoclonal and polyclonal antibodies, as well as chapters describing ways of using antibodies to study antigens. Two biases are clear: we have concentrated on protein antigens, and we have excluded several of the classical methods of immunochemistry. In their place, we have concentrated on protocols for cell staining, immunoprecipitation, immuno-

blotting, immunoaffinity purification, and immunoassay, as these are the techniques most commonly required by the nonimmunologist.

The actual origin of a protocol is often hard to determine, and although we have given appropriate references wherever possible, there are many sins of omission. The methods have been derived mostly from those used in our labs or those of our close associates. Clearly, few protocols are definitive, and this is particularly true where so much depends on the individual qualities of a particular antibody. We have included both general notes on the procedures themselves plus more personalized comments. These are distinguished by different types of "boxes" in the text. The phrase "some workers find..." is used either when we could not reach an agreement among ourselves or when friends assured us the effect was significant but neither of us had any direct experience with it. We have attempted to give a clear explanation of the theoretical and practical basis of the techniques to provide an effective guide when problems arise.

For further general information, *Handbook of Experimental Immunology* edited by Weir, Herzenberg, Blackwell, and Herzenberg (1986) is the best source of technical advice, and *Monoclonal Antibodies: Principles and Practice* by Goding (1987) and *Hybridoma Technology in the Biosciences and Medicine* edited by Springer (1985) are excellent sources of information on monoclonal antibodies. More specific references are listed in the appropriate sections. For new protocols, the reader should scan the pages of the *Journal of Immunological Methods* and *Analytical Biochemistry*.

Many, many people helped us with these protocols, and our job has often been one of collation, checking, and interpretation. We would like to thank Joan Brugge and her lab (Michael DeMarco, Adele Filson, Lawrence Fox, Andy Golden, Joan Levy, Sally Lynch, Susan Nemeth, John Schmidt, and Susan Schuh) who used an early version of the manual and were immensely helpful, both in finding fault and in making powerful suggestions for improvement. Several members of our own labs, Carmelita Bautista, Karen Buchkovich, Margaret Falkowski, Julian Gannon, Richard Iggo, Margaret Raybuck, and Carmella Stephens, used the manual on a day-to-day basis and were very definite and precise in their criticisms!

The manual was read in its entirety by Lionel Crawford, Larry Banks, Mike Krangel, Margaret Raybuck, and David Chiswell and his colleagues at Amersham. Their many comments helped us resolve difficult decisions about content and presentation. Chapters 1–4 were read and critiqued by Winship Herr, Richard Iggo, and John Inglis. Steve Dilworth, Ann Harris, and Bob Knowles sorted out Chapters 6–9 and 15, and Jean Beggs and Birgitte Lane helped enormously to get Chapters 10–14 into shape. The appendices were read and amended by Carl Anderson and Mark Zoller. In addition to these main readers, many others kindly helped with specific sections. Advice on yeast came from John Kilmartin, Jean Beggs, Paul Nurse, and David Beach. Advice from Susan Alpert on cell staining was invaluable, as were Rebbeca Rowehl's and Jaqueline Bortzner's comments on Chapters 6 and 7. Ian Mohr forced us to get the immunoassay chapter into

intelligible form, Seth Grant helped on Chapter 12, and Ella Wetzel read and reviewed an early version of Chapter 11. All these people helped to make this a better book. Usually, we took their advice, and we hope they will forgive us for those times that we didn't.

Special personal thanks to Birgitte Lane, Nicky Williamson, Gordon Peters, Brenda Marriot, Frank Fitzjohn, and Marilyn Goodwin who helped by getting us onto planes at the right time, by finding us places to sleep and write, and by fending off a wide range of tempting distractions. Somehow they managed still to be nice, even when we were at our most crabby. The excellent art work is by Mike Ockler, and looking at Carl Molno's suggestions for cover art was a wonderful break. Jim Pflugrath provided the computer graphics. Christy Kuret, Michele Ferguson, Inez Sialiano, and Susan Schaefer provided invaluable editorial assistance. The book was designed by Emily Harste (despite our interference), and Nancy Ford and Annette Kirk provided overall supervision and kind support throughout the work.

A final and special thanks to Judy Cuddihy, our editor, whose patient advice, encouragement, and fine judgement kept us going through the best and worst of times.

Hope it's fun,

EH and **DL**

6

Serum contains many different types of antibodies that are specific for many different antigens. Even in hyperimmune animals, seldom are more than one-tenth of the circulating antibodies specific for one antigen. The use of these mixed populations of antibodies creates a variety of different problems in immunochemical techniques. Therefore, the preparation of homogeneous antibodies with a defined specificity was a long-standing goal of immunochemical research. This goal was achieved with the development of the technology for hybridoma production.

The first isolation of a homogeneous population of antibodies came from studies of B-cell tumors. Clonal populations of these cells can be propagated as tumors in animals or grown in tissue culture. Because all of the antibodies secreted by a B-cell clone are identical, these tumor cells provide a source of homogeneous antibodies. Unfortunately, B-cell tumors secreting antibodies of a predefined specificity cannot be isolated conveniently.

In the animal, antibodies are synthesized primarily by plasma cells, a type of terminally differentiated B lymphocyte. Because plasma cells cannot be grown in tissue culture, they cannot be used as an in vitro source of antibodies. Köhler and Milstein (1975) developed a technique that allows the growth of clonal populations of cells secreting antibodies with a defined specificity. In this technique an antibody-secreting cell, isolated from an immunized animal, is fused with a myeloma cell, a type of B-cell tumor. These hybrid cells or *hybridomas* can be maintained in vitro and will continue to secrete antibodies with a defined specificity. Antibodies that are produced by hybridomas are known as *monoclonal antibodies*.

Monoclonal antibodies are powerful immunochemical tools

The usefulness of monoclonal antibodies stems from three characteristics–their specificity of binding, their homogeneity, and their ability to be produced in unlimited quantities. The production of monoclonal antibodies allows the isolation of reagents with a unique, chosen specificity. Because all of the antibodies produced by descendants of one hybridoma cell are identical, monoclonal antibodies are powerful reagents for testing for the presence of a desired epitope. Hybridoma cell lines also provide an unlimited supply of antibodies. Even the most farsighted researchers have found that large supplies of valuable antisera eventually run out. Hybridomas overcome these difficulties. In addition, one unique advantage of hybridoma production is that impure antigens can be used to produce specific antibodies. Because hybridomas are single-cell cloned prior to use, monospecific antibodies can be produced after immunizations with complex mixtures of antigens.

Hybridomas secreting monoclonal antibodies specific for a wide range of epitopes have been prepared. Any substance that can elicit a humoral response can be used to prepare monoclonal antibodies. Their

specificities range from proteins to carbohydrates to nucleic acids. However, monoclonal antibodies are often more time-consuming and costly to prepare than polyclonal antibodies, and they are not necessarily the best choice for certain immunochemical techniques. In theory, either as single antibody preparations or as pools, monoclonal antibodies can be used for all of the tasks that require or benefit from the use of polyclonal antibodies. In practice, however, producing exactly the right set of monoclonal antibodies is often a difficult and laborious job. Researchers should be certain that they need these types of reagents before they begin constructing hybridoma cell lines. Table 6.1 summarizes some of the uses of antibodies and some general suggestions for choosing the best reagents.

Hybridomas are immortal somatic cell hybrids that secrete antibodies

In the early 1970s, a number of research groups worked on different methods to extend the life span of antibody-secreting cells in vitro. For murine cells, the practical aspects of this goal were solved by applying techniques used in somatic cell genetics. By fusing two cells, each having properties necessary for a successful hybrid cell line, Köhler and Milstein (1975) showed that antibody-secreting cell lines could be established routinely and maintained in vitro. The two cells that are commonly used as partners in these fusions are antibody-secreting cells isolated from immunized animals and myeloma cells. The myeloma cells provide the correct genes for continued cell division in tissue culture, and the antibody-secreting cells provide the functional immunoglobulin genes.

Early work solved the three technical problems for achieving a successful fusion: (1) finding appropriate fusion partners, (2) defining conditions for efficient fusion, and (3) choosing an appropriate system to select for hybrid cells against the background of unfused cells.

TABLE 6.1
Immunochemical Techniques, Polyclonal versus Monoclonal Antibodies

Technique	Polyclonal antibodies	Monoclonal antibodies	Pooled monoclonal antibodies
Cell Staining	Usually good	Antibody dependent	Excellent
Immunoprecipitation	Usually good	Antibody dependent	Excellent
Immunoblots	Usually good	Antibody dependent	Excellent
Immunoaffinity Purification	Poor	Antibody dependent	Poor
Immunoassays			
Labeled Antibody	Difficult	Good	Excellent
Labeled Antigen	Usually good	Antibody dependent	Excellent

Myelomas from BALB/c mice are good cells for fusion

Myelomas can be induced in a few strains of mice by injecting mineral oil into the peritoneum. Many of the first examples of these myelomas were isolated from BALB/c mice by Potter (1972), and these cells are referred to by the abbreviation MOPC (for mineral oil plasmacytoma). Derivatives of BALB/c myelomas have become the most commonly used partners for fusions. Table 6.2 lists some of the myeloma cell lines used for hybridoma construction. Myelomas have all the cellular machinery necessary for the secretion of antibodies, and many secrete these proteins. To avoid the production of hybridomas that secrete more than one type of antibody, myelomas that are used for fusions have been selected for the lack of production of functional antibodies. Figure 6.1 shows the derivation of many of the commonly used myeloma cell lines.

The other cell for the fusion is isolated from immunized animals. These cells must carry the rearranged immunoglobulin genes that specify the desired antibody. Because of the difficulties in purifying cells that can serve as appropriate partners, fusions are normally performed with a mixed population of cells isolated from a lymphoid organ of the immunized animal. Although a number of studies have helped to characterize the nature of this B-cell-derived partner, the exact state of differentiation of this cell is still unclear.

Hybridomas can be prepared by fusing myelomas and antibody-secreting cells isolated from different species, but the number of viable hybridomas increases dramatically when closely related species are used. Therefore, fusions are normally done with cells from the same species. All commonly used mouse strains can serve as successful fusion partners with BALB/c myelomas; however, immunizations are normally done in BALB/c mice, as this allows the resulting hybridomas to be grown as tumors in this mouse strain.

Polyethylene glycol is the most commonly used agent to fuse mammalian cells

In theory, the fusion between the myeloma cell and the antibody-secreting cell can be effected by any fusogen. In practice, hybridoma fusions became routine after the introduction of the use of polyethylene glycol (PEG). The use of PEG as a fusing agent for mammalian cells was first demonstrated by Pontecorvo (1975), and was quickly adopted by somatic cell geneticists. PEG is the method of choice for hybridoma production, allowing the rapid and manageable fusion of mammalian cells.

PEG fuses the plasma membranes of adjacent myeloma and/or antibody-secreting cells, forming a single cell with two or more nuclei. This heterokaryon retains these nuclei until the nuclear membranes dissolve prior to mitosis. During mitosis and further rounds of division, the individual chromosomes are segregated into daughter cells.

TABLE 6.2
Myeloma Cell Lines Used as Fusion Parents

Cell line	Reference	Derived from	Chains expressed	Secreting	Comments
Mouse Lines					
P3-X63Ag8	Köhler and Milstein (1975)	P3K	$\gamma 1, \kappa$	IgG_1	Not recommended
X63Ag8.653	Kearney et al. (1979)	P3-X63Ag8	None	No	Recommended
Sp2/0-Ag14	Köhler and Milstein (1976) Shulman et al. (1978)	P3-X63Ag8 × BALB/c	None	No	Recommended
FO	de St. Groth and Scheidegger (1980)	Sp2/0-Ag14	None	No	Recommended
NSI/1-Ag4-1	Köhler et al. (1976)	P3-X63Ag8	Kappa	No	Recommended
NSO/1	Galfre and Milstein (1981)	NSI/1-Ag4-1	None	No	Recommended
FOX-NY	Taggart and Samloff (1984)	NSI/1-Ag4-1	Kappa (?)	No	
Rat Lines					
Y3-Ag1.2.3	Galfre et al. (1979)	Y3	Kappa	No	Not recommended
YB2/0	Kilmartin et al. (1982)	YB2/3HL	None	No	Recommended
IR983F	Bazin (1982)	LOU/c rats	None	No	Recommended

Because of the abnormal number of chromosomes, segregation does not always deliver identical sets of chromosomes to daughter cells, and chromosomes may be lost. If one of the chromosomes that carries a functional, rearranged immunoglobulin heavy- or light-chain gene is lost, production of the antibody will stop. In a culture of hybridoma cells, this will be seen phenotypically as a decrease in antibody titer and will result in unstable lines. If the chromosome that is lost contains a gene used in drug selection (see below), then the growth of the hybridoma will be unstable, and cells will continue to die during selection. In practice, the selection for the stable segregation of the drug selection marker is so strong that within a short time the hybridoma is either lost completely or a variant is isolated that stably retains the selectable marker.

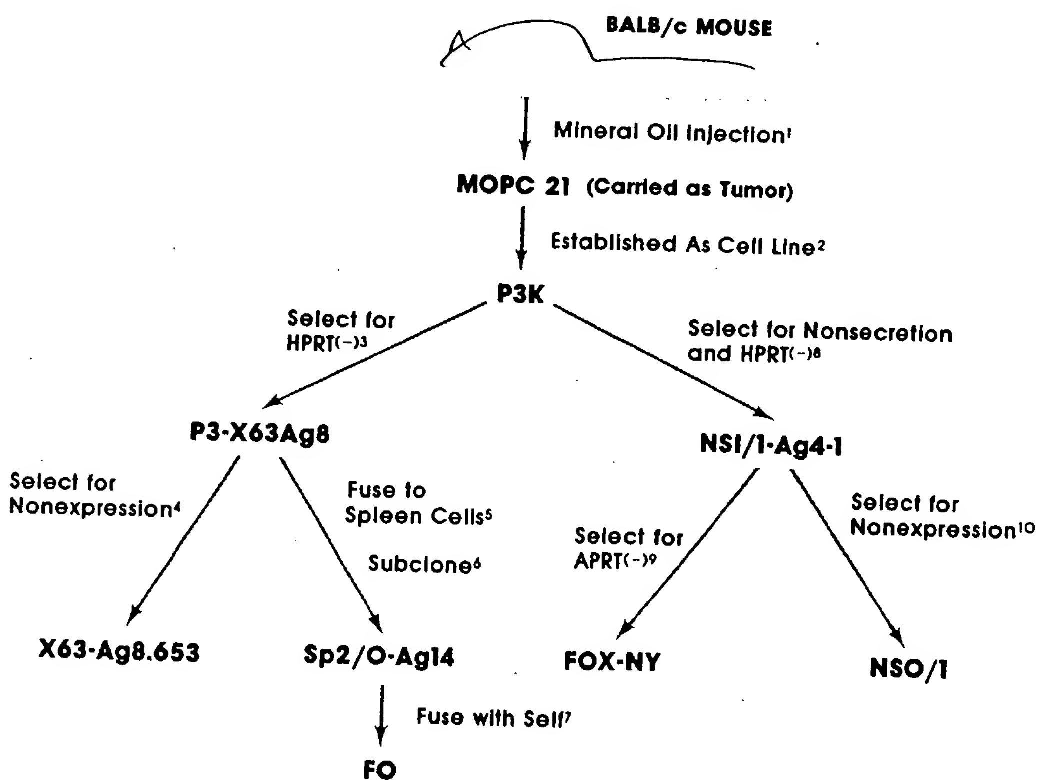


FIGURE 6.1
Myeloma family tree. [1]Potter (1972); [2]Horibata and Harris (1970); [3]Kohler and Milstein (1975); [4]Kearney et al. (1979); [5]Kohler and Milstein (1975); [6]Shulman et al. (1978); [7]de St. Groth and Scheidegger (1980); [8]Kohler et al. (1976); [9]Taggart and Samloff (1982); [10]Galfre and Milstein (1981).

Unfused myeloma cells are eliminated by drug selection

Even in the most efficient hybridoma fusions, only about 1% of the starting cells are fused, and only about 1 in 10^5 form viable hybrids. This leaves a large number of unfused cells still in the culture. The cells from the immunized animal do not continue to grow in tissue culture, and so do not confuse further work. However, the myeloma cells are well adapted to tissue culture and must be killed. Most hybridoma constructions achieve this by drug selection. Commonly, the myeloma partner has a mutation in one of the enzymes of the salvage pathway of purine nucleotide biosynthesis (first reported by Littlefield 1964). For example, selection with 8-azaguanine often yields a cell line harboring a mutated hypoxanthine-guanine phosphoribosyl transferase gene (HPRT). The addition of any compound that blocks the de novo nucleotide synthesis pathway will force cells to use the salvage pathway. Cells containing a nonfunctional HPRT protein will die in these conditions. Hybrids between myelomas with a nonfunctional HPRT and cells with a functional HPRT will be able to grow. Selections are commonly done with aminopterin, methotrexate, or azaserine (Fig. 6.2).

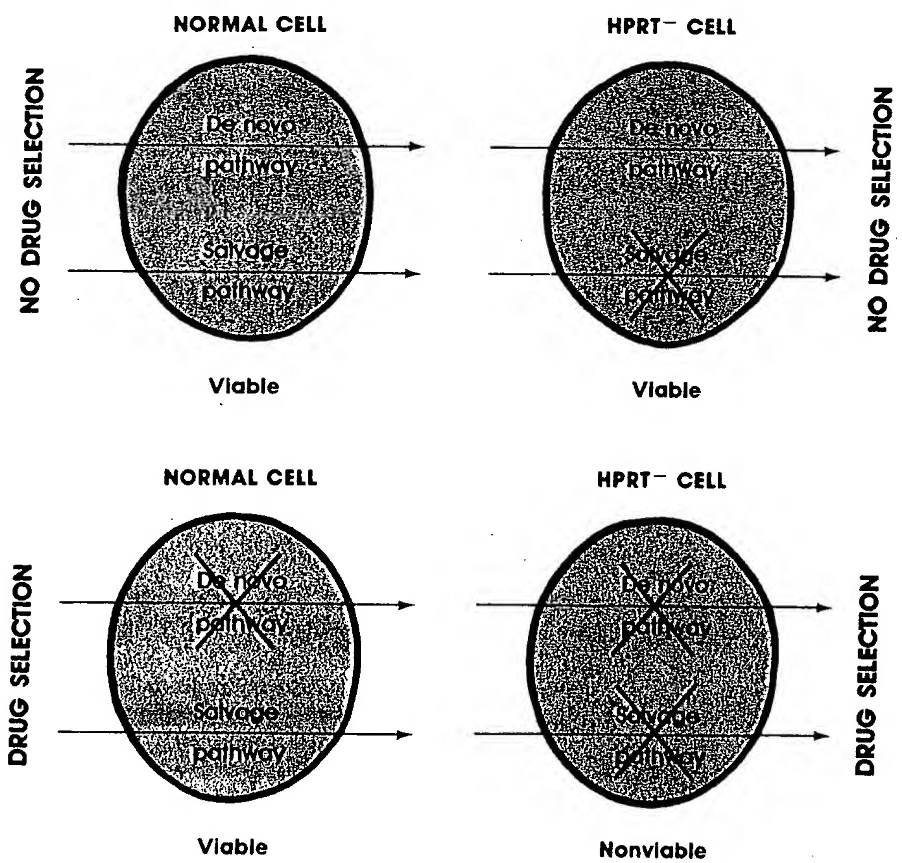


FIGURE 6.2A
Pathways of nucleotide synthesis.

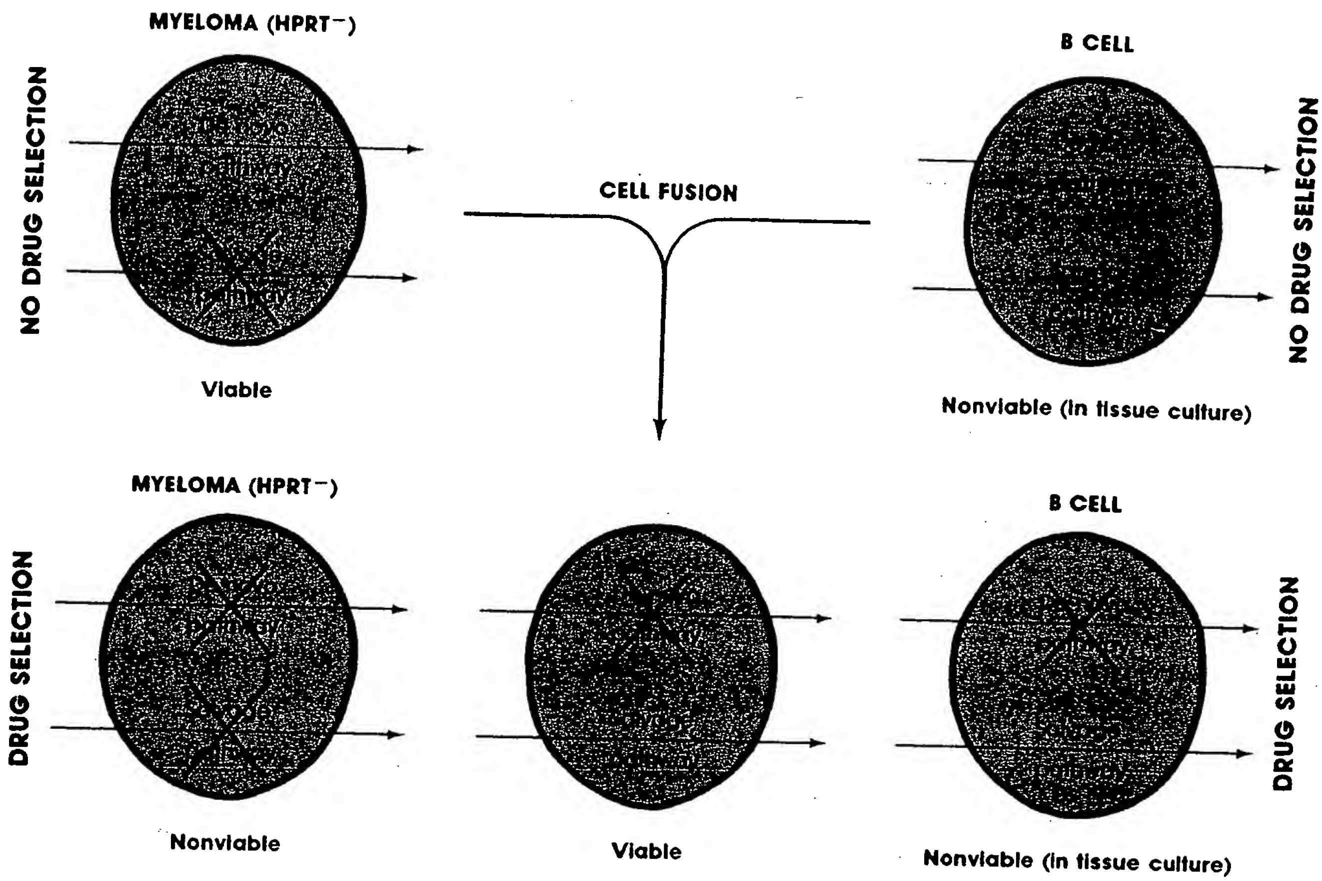


FIGURE 6.2B
Drug selection for viable hybridomas.

PRODUCTION OF MONOCLONAL ANTIBODIES

Although hybridomas can be prepared from animals other than mice, all of the techniques below use mice as examples. Similar techniques can be used for fusions of rat myelomas and rat antibody-secreting cells. More specialized fusions using interspecies crosses or human cells are discussed briefly on p. 240 and 241, respectively.

Stages of Hybridoma Production

Figure 6.3 outlines the steps in the production of monoclonal antibodies. Animals are injected with an antigen preparation, and once a good humoral response has appeared in the immunized animal, an appropriate screening procedure is developed. The sera from test bleeds are used to develop and validate the screening procedure. After an appropriate screen has been established, the actual production of the hybridomas can begin. Several days prior to the fusion, animals are boosted with a sample of the antigen. For the fusion, antibody-secreting cells are prepared from the immunized animal, mixed with the myeloma cells, and fused. After the fusion, cells are diluted in selective medium and plated in multiwell tissue culture dishes. Hybridomas are ready to test beginning about 1 week after the fusion. Cells from positive wells are grown and then single-cell cloned. Hybridoma production seldom takes less than 2 months from start to finish, and it can take well over a year. It is convenient to divide the production of monoclonal antibodies into three stages: (1) immunizing mice, (2) developing the screening procedure, and (3) producing hybridomas. Any one of these stages may proceed very quickly, but all have inherent problems that should be considered prior to the start of the project, and these areas are discussed separately below.

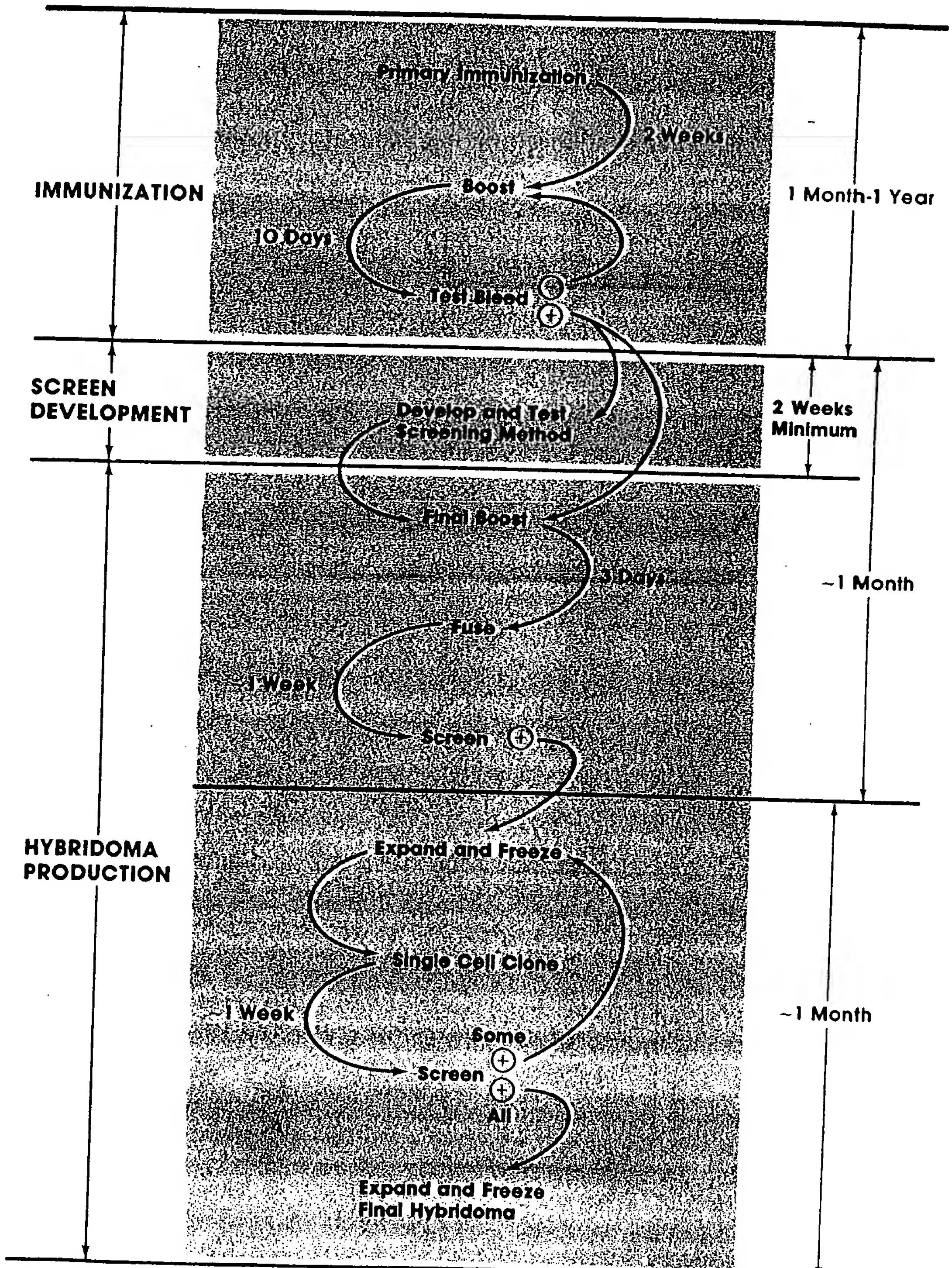


FIGURE 6.3
Stages of hybridoma production.

IMMUNIZING MICE

Figure 6.4 shows a typical antibody response to multiple injections with a good immunogen. Also included in this figure is a description of the characteristics of a typical monoclonal antibody that might be isolated following one of the immunizations. Although this simplified view can only serve as a rough guide, it does give an indication of the potential time frame for the production of antibodies with particular properties.

Chapters 4 and 5 discuss in detail both the theoretical and practical aspects of immunizing laboratory animals.

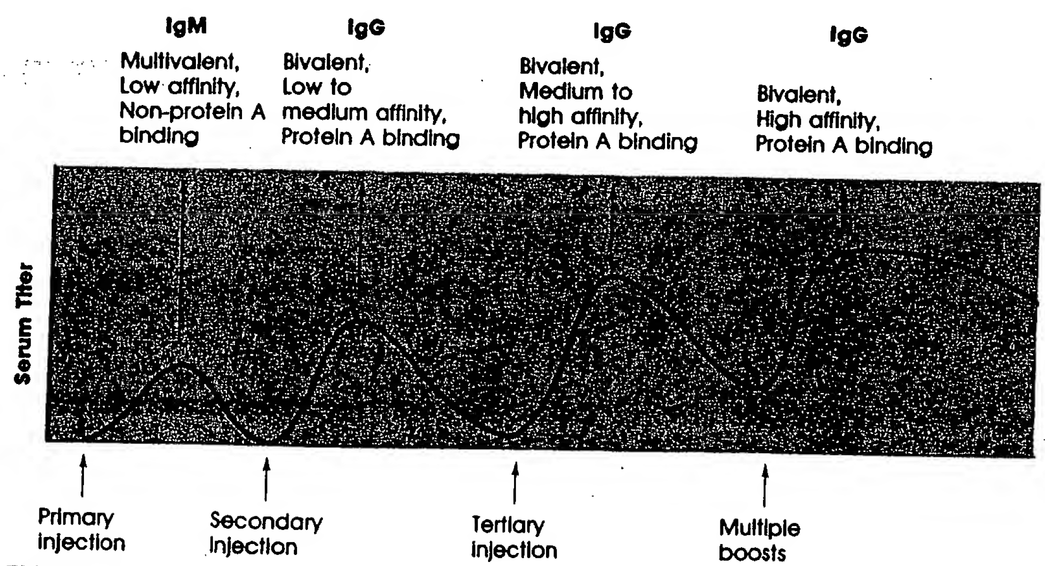


FIGURE 6.4
Kinetics of a typical immune response.

Dose and Form of the Antigen

The amount of antigen necessary to induce a good immune response will depend on the individual antigen and host animal (see Chapter 5). Suggested doses for mice are summarized in Table 6.3.

Soluble Proteins

Soluble protein antigens can yield strong responses and good monoclonal antibodies with doses of as low as 1 μg/injection. More commonly, injections are adjusted to deliver 10–20 μg. If the antigen is available in large quantities, 50 μg should be used. Except for special cases it is seldom worthwhile to use more than 200 μg of a protein antigen per injection. Even if the antigen is not pure, the total dose should not normally exceed 500 μg. When highly conserved proteins are being used to raise antibodies, it is often necessary to modify these antigens prior to injection. This can be done by covalently adding small immunogenic haptens to proteins. Modifying proteins by binding them to large immunogenic proteins such as the hemocyanins has also been shown to be an effective way of breaking T cell tolerance. These methods are discussed in detail in Chapter 5.

Example of a Typical Immunization Schedule

1. [illegible] mix 250 μl of antigen solution with 250 μl of complete Freund's [illegible] BALB/c female mice [illegible].
2. [illegible] days repeat the injections but use incomplete Freund's adjuvant.
3. Collect tail bleeds from immunized mice on day 24. Do 1 in 5 dilution in PBS and [illegible] samples by comparison with similar dilutions of normal mouse serum in a [illegible]
4. [illegible] all animals ip with incomplete Freund's.
5. Day [illegible] tail bleeds and test by dot blot. All serum samples checked by immunoprecipitation against in vivo radiolabeled antigen preparation.
6. Day 56 inject best responder, 100 μl iv and 100 μl ip. All others get ip injection with incomplete Freund's.
7. Day 59, fuse splenocytes from best responder.
8. Day 60, hard work starts, but champagne for first positives.

TABLE 6.3
Suggested Doses of Immunogens for Mice

Form of antigen	Examples	Primary injections and boosts			Final boosts		
		Possible routes	Dose	Adjuvant	Possible routes	Dose	Adjuvant
Soluble Proteins	Enzymes Carrier proteins conjugated with peptides Immune complexes	ip[a] sc[b]	5–50 μg	+	iv[c]	5–50 μg	–
Particulate Proteins	Viruses (killed) Yeast (killed) Bacteria (killed) Structural proteins	ip sc	5–50 μg	+	iv	5–50 μg	–
Insoluble Proteins	Bacterially produced from inclusion bodies Immunopurified proteins bound to beads	ip sc	5–50 μg	+	ip	5–50 μg	–
Live Cells	Mammalian cells	ip	10^5–10^7 cells	–	iv	10^6 cells	–
Live Tumorigenic Cells	Oncogenic mammalian cells	ip sc	10^4–10^6 cells	–	iv	10^6 cells	–
Carbohydrates	Polysaccharides Glycoproteins	ip sc	10–50 μg	+/–	iv	10–50 μg	–
Nucleic Acids	Carrier proteins conjugated with N.A.	ip	10–50 μg	+	iv	10–50 μg	–

[a]Intraperitoneal.
[b]Subcutaneous.
[c]Intravenous.

Particulate Proteins

In general, particulate antigens make excellent immunogens, because they are readily phagocytosed (see Chapter 4). Soluble proteins may be converted to particulate antigens by self-polymerization or by binding them to solid substrates such as agarose beads (p. 528). Large insoluble antigens should not be injected intravenously (iv) due the possible development of embolisms.

Proteins Produced by Overexpression

Recent advances in recombinant DNA technology have made the production of many protein antigens simple. Overexpression of fusion proteins or full-length polypeptide chains using both prokaryotic and eukaryotic vectors has become routine. These proteins are often excellent antigens and can be produced in large quantities. They normally present few problems for the production of monoclonal antibodies. These proteins can be purified and injected as soluble or insoluble antigens (p. 88).

Synthetic Peptides

A second source of immunogens, based on the availability of a coding sequence, is the in vitro synthesis of peptides. Synthetic peptides, when coupled to carrier proteins such as bovine serum albumin or keyhole limpet hemocyanin, normally elicit a strong humoral response. Constructing these carrier complexes and the production of anti-peptide sera are described in Chapter 5 (p. 73). Using peptide–carrier protein complexes for the production of monoclonal antibodies normally is done only for specific reasons. Because these peptides are relatively short, many of the advantages of monoclonal antibody specificity are lost. Monoclonal antibodies do provide two advantages over polyclonal anti-peptide sera. The first is that the source of the antibodies will be unlimited, and the second is that monoclonal antibodies may be more useful in immunoaffinity purifications. Like all immunizations using peptide antigens, the major difficulty will be in preparing antibodies that will bind to the native protein.

Live Cells

A number of studies have used live cells as immunogens for generating antibodies to surface antigens. Except in unusual circumstances, injections of cells should not include live bacteria or yeast. Although mice are normally capable of killing and clearing bacteria and yeast infections, the possibility of infecting an entire mouse colony is too great to risk these types of injections.

Although large numbers of hybridomas have been prepared to surface antigens of mammalian cells, these antibodies may be of low affinity and care should be taken to ensure that the immune response includes antibodies that will be useful in later studies. When raising

antibodies to live tumorigenic cells, it is easy to pass the cells as tumors and thereby eliminate any activity against tissue culture reagents, including proteins in bovine serum.

Nucleic Acids

Nucleic acids normally are not good antigens, and antibodies to them usually are raised against small haptens bound to carrier proteins. Because nucleic acids are weak antigens, it is particularly important to test sera for antibodies that will work in all assays for which the monoclonal antibodies are being raised.

Carbohydrates

Simple carbohydrates usually are weak immunogens. These compounds should be coupled to carrier proteins. Large complex carbohydrates (> 50,000) will induce a moderate response, but often without a secondary response. High doses readily induce tolerance, so the injected amount should be controlled carefully. These immunogens are best injected as a portion of a larger particle, such as a bacterial cell wall

TABLE 6.4
Routes of Injection

Route	Abbreviation	Uses: Primary injections and boosts	Uses: Final boost	Maximum volume
Intraperitoneal	ip	Good	Fair	0.5 ml
Subcutaneous	sc	Good	Poor	0.2 ml
Intravenous	iv	Poor	Good	0.2 ml
Intramuscular	im	Not recommended for mice		
Intradermal	id	Not recommended for mice		
Lymph node		Special uses		0.1 ml

or equivalent. Coupling these larger carbohydrates to carrier proteins can be beneficial. For glycoproteins, the polypeptide backbone can function as an effective carrier.

Route of Inoculation

Table 6.4 gives a summary of the potential routes of introducing an antigen into mice. Most injections for hybridoma production are done in female mice, because they are somewhat easier to handle than male mice.

> Prior to beginning an immunization, contact your local safety and animal committees for advice on animal care and handling, local regulations, and proper procedures for immunization.

Adjuvant	Immunogen requirement	Comments	Route
+/−	Soluble/or insoluble	If used for final boost, wait 5 days before fusion	**Intraperitoneal**
+/−	Soluble/or insoluble	Local response, Serum levels slower to increase	**Subcutaneous**
No Freund's	Soluble, Ionic detergent <0.2% Nonionic detergent <0.5% Salt <0.3 M Urea <1 M	Poor for immunizing	**Intravenous**
			Intramuscular
			Intradermal
No Freund's	Soluble/or insoluble	Good applications for experienced workers	**Lymph node**

COMMENTS ■ Immunizations

There are several points that are important in designing an immunization regime that will produce the appropriate monoclonal antibodies.

- Choose the appropriate animal or strain for the desired antibody. Important points to consider are (1) tolerance, (2) amount of antigen available, and (3) specific properties (including ease of purification) of the resultant antibodies. (See Chapters 4 and 5 for details.)

  If no preference in the choice of animal is dictated, then start the immunizations in female BALB/c mice (6 weeks old). In general, mice are cheaper to maintain, are easier to handle, and will respond to lower antigen levels than other laboratory animals. In addition, BALB/c × BALB/c hybridomas can be grown as ascites in BALB/c mice (see p. 274). This can be valuable both in the production of large quantities of monoclonal antibodies and in the eradication of contaminating microorganisms from cultures of hybridoma cells grown in vitro.

- Individual animals, even from the same genetic background, will often respond to identical antigen preparations in completely different ways. Therefore, immunizing more than one animal is a major advantage. In addition, laboratory animals occasionally die, so starting immunizations with several animals may save valuable time.

- Hyperimmunization (multiple immunizations with the same antigen) will yield antibodies with higher affinity for the antigen, especially when the immunizations are widely spaced over a period of weeks to months (see Chapter 5). However, multiple immunizations will not continue to increase the number of epitopes that are recognized.

- Except in unusual circumstances, **do not** start the fusion until the serum from the test bleed contains antibodies with the desired specificity. This may mean extensive testing of the serum in a number of assays, but do not expect to recover antibody activities from the fusion that are not found in the test serum.

- If the animal responds weakly or not at all, consult Chapter 5 for suggestions.

INTRAPERITONEAL INJECTIONS—WITH ADJUVANT

Intraperitoneal injections (ip) are the most commonly used method for introducing antigens into mice. Because of the large volume of the peritoneal cavity, the volume of the immunogen can be larger for ip injections than for other sites. Also because the injections do not deliver the antigen directly into the blood system, particulate antigens can be used.

> **Caution** Freund's adjuvants are potentially harmful. Be careful during preparation and injection.

1. The antigen should be dispersed in approximately 250 μl of buffer. Draw the solution into a 1.0-ml syringe. Draw an equal volume of Freund's adjuvant into a second syringe. For a primary immunization the adjuvant should be complete Freund's (see p. 98). Complete Freund's should be vortexed before use to resuspend the killed *Mycobacterium tuberculosis* bacteria. All other injections should be in incomplete Freund's.

2. Aqueous antigen solutions and oil-based adjuvants are immiscible, but when mixed will form an emulsion. This is most easily done by pushing the mixture between the two 1-ml syringes via a two- or three-way valve as shown in Figure 6.5. Remove all air from both

FIGURE 6.5
Preparing Freund's adjuvant for injection.

syringes before connecting to the valve. Make sure the connections are tightened securely. Depress the plunger on the syringe containing the aqueous antigen solution first. Push the mixture between the two syringes until it becomes difficult to continue (approximately 10–20 times). Then push the emulsion into one syringe. Remove the valve and the other syringe, and place a 23- or 25- gauge needle on the syringe. Displace any air that may be held in the barrel or in the needle by carefully depressing the plunger until the emulsion reaches the needle.

3. Hold the mouse as shown in Figure 6.6. Inject the antigen–adjuvant emulsion into the peritoneal cavity.

NOTES

i. If the volume of the antigen solution is small (100 μl or less), the emulsion between the Freund's and aqueous solutions may be prepared by vortexing or sonicating in a 1.5-ml conical tube.

ii. Plastic syringes may be difficult to use when preparing the emulsion; therefore, most workers use glass syringes (glass disposable syringes with luer locks are best).

iii. Freund's adjuvants can be replaced by other adjuvants (see p. 96).

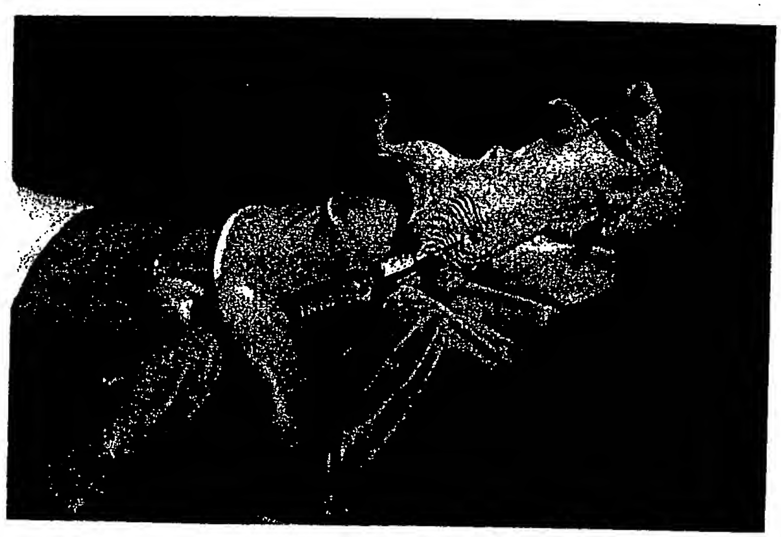


FIGURE 6.6
Intraperitoneal injection of a mouse.

INTRAPERITONEAL INJECTIONS—WITHOUT ADJUVANTS

Intraperitoneal injections can also be used to deliver live cells into the peritoneal cavity. These immunizations normally are used to introduce live mammalian cells into mice and prepare anti–cell-surface antibodies. In general, adjuvants should not be used.

1. Cells should be washed extensively in PBS or other isotonic solutions prior to injection to remove as many extraneous proteins as possible. For example, many of the components of bovine serum are highly immunogenic, and if they are injected with the cells, can be antigenically dominant. Even washed cells will have a large number of extraneous proteins bound to the plasma membrane. If this remains a problem, it may be necessary to transfer the cells to low serum, serum-free medium, or mouse serum prior to the injection.

2. The cells should be taken up in 500 μl of PBS and injected using a 25-gauge needle. Hold the mouse as shown in Figure 6.6.

3. Inject the cells into the peritoneum.

NOTES

i. Normal doses of mammalian cells will be between 10^5 and 10^7 cells/injection.

ii. Because of potential infections of the mouse colony, injections of live viruses, bacteria, or fungi normally are not recommended. These antigens are commonly killed or inactivated prior to injection.

INTRAPERITONEAL INJECTION—ANTIGEN BOUND TO BEADS

One excellent method to increase the chances of an antigen being phagocytosed is to bind it to beads. Protein antigens can be bound by free amino groups to any of a number of agarose or polyacrylamide beads. The methods for coupling are discussed on p. 528. After coupling the beads are injected ip with adjuvant as described on p. 158. These preparations should never be injected iv because the chance of forming embolisms is too great.

INTRAPERITONEAL INJECTION—NITROCELLULOSE

Protein antigens can be bound to nitrocellulose and injected or implanted into the peritoneum. Two approaches can be used. If the entire piece of nitrocellulose will be implanted, the subcutaneous route is suggested (see p. 166). For intrapertioneal injections, the nitrocellulose should either be fragmented or dissolved.

> **Caution** Freund's adjuvants are potentially harmful. Be careful during preparation and injection.

1. Incubate a solution of the antigen (no more than 1.0 mg/ml in PBS) with a sheet of nitrocellulose (0.1 ml/cm^2) at room temperature for 1 hr in a humid atmosphere. For some more abundant antigens or partially purified antigens, the proteins can be transferred directly from an SDS-polyacrylamide gel using standard blotting techniques (see p. 479).

2. Wash the sheet three times with PBS.

3. **Either**: Drain the paper well and freeze at −70°C for 10 min (liquid nitrogen also works well). Transfer to a clean, cold mortar and pestle and quickly grind the paper into small pieces. Remove the plunger from a 1.0-ml syringe, and transfer the pieces into the barrel. Use 250 μl of PBS to help in the transfer.

   Or: Allow the nitrocellulose to dry completely. Remove the plunger from a 1.0-ml syringe. Push the nitrocellulose (use pure nitrocellulose without acetate) to the bottom of barrel. Reinsert the plunger and depress completely. Draw up 125 μl of dimethylsulfoxide into the syringe. Alllow to sit at room temperature for 30 min. Draw up 125 μl of PBS.

   Or: Allow the nitrocellulose to dry completely. Remove the plunger from a 1.0-ml syringe. Push the nitrocellulose (use pure nitrocellulose without acetate) to the bottom of the barrel. Push the luer lock of the syringe into a three-way valve with the valve opening to the syringe closed. Add 250 μl of acetone to the syringe. Tap the syringe several times to mix the acetone with the nitrocellulose. Leave the syringe open and allow to dry (several hours). Remove the three-way valve and replace the plunger. Push the plunger to the end and draw 250 μl of PBS into the syringe.

4. Draw 250 μl of Freund's adjuvant into a second syringe. For a primary immunization the adjuvant should be complete Freund's. (Complete Freund's should be vortexed before use to resuspend the killed *Mycobacterium tuberculosis* bacteria.) All other injections should be in incomplete Freund's.

5. The nitrocellulose mixture and the oil-based adjuvant are immiscible, but when mixed will form an emulsion. This is most easily done by pushing the mixture between the two 1-ml syringes via a two- or three-way valve as shown in Figure 6.5. Remove all air from both syringes before connecting to the valve. Make sure the connections are tightened securely. Depress the plunger on the syringe containing the PBS solution first. Continue to push the mixture between the two syringes until it becomes difficult (approximately 10–20 times). Push the emulsion into one syringe. Remove the valve and the other syringe, and place a 23- to 25-gauge needle on the syringe. Displace any air that may be held in the barrel or in the needle by carefully depressing the plunger until the emulsion reaches the needle.

6. Hold the mouse as shown in Figure 6.6. Inject the antigen–adjuvant emulsion into the peritoneal cavity.

NOTES

i. Plastic syringes may be difficult to use when preparing the emulsion; therefore, most workers use glass syringes (glass disposable syringes with luer locks are best).

ii. Freund's adjuvants can be replaced by other adjuvants (see p. 96).

SUBCUTANEOUS INJECTIONS—WITH ADJUVANTS

Subcutaneous injections (sc) are used to deliver soluble or insoluble antigens into a local environment that is a good site of lymphoid draining. Maximum volumes for sc injections are about one-fifth the maximum used for ip injections (100 μl compared to 500 μl). Subcutaneous injections normally are done at more than one site to help ensure that the antigen is detected.

Caution Freund's adjuvants are potentially harmful. Be careful during preparation and injection.

1. The antigen should be dispersed in approximately 50–100 μl per site of injection. Take up into a 1.0-ml syringe. Take up an equal volume of Freund's adjuvant into a second syringe barrel. For a primary immunization the adjuvant should be complete Freund's. (Complete Freund's should be vortexed before use to resuspend the killed *Mycobacterium tuberculosis* bacteria.) All other injections should be in incomplete Freund's.

2. The adjuvant, which is oil based, and the aqueous antigen solutions are immiscible, but with mixing will form an emulsion. This is most easily done by pushing the mixture between the two 1-ml syringes via a two- or three-way valve as shown in Figure 6.5. Remove all air from both syringes before connecting to the valve. Make sure the connections are securely tightened. Depress the plunger on the syringe containing the aqueous antigen solution first. Continue to push the mixture between the two syringes until it becomes difficult to push (approximately 10–20 times). Push the emulsion into one syringe. Remove the valve and the other syringe, and place a 23- or 25-gauge needle on the syringe. Displace any air that may be held in the barrel or in the needle by carefully depressing the plunger until the emulsion reaches the needle.

3. Hold the mouse as shown in Figure 6.7. Inject approximately 200 μl total under the skin.

NOTES

i. If the volume of the antigen solution is small (100 μl or less), the emulsion between the Freund's and aqueous solutions may be prepared by vortexing or sonicating in a 1.5-ml conical tube.

ii. Plastic syringes may be difficult to use when preparing the emulsion; therefore, most workers use glass syringes (glass disposable syringes are best).

iii. Freund's adjuvants can be replaced by other adjuvants (see p. 96).

SUBCUTANEOUS INJECTIONS—WITHOUT ADJUVANTS

Like the peritoneal injections, subcutaneous (sc) injections that do not use adjuvants normally are used for delivering live cells to the mouse. This route is often used for tumorigenic cells.

1. Cells should be washed carefully prior to injection to remove proteins from the growth medium. For example, many of the components of bovine serum are highly immunogenic, and, if they are injected with the cells, they can be antigenically dominant. Even washed cells will have a large number of extraneous proteins bound to the plasma membrane. If this remains a problem, it may be necessary to transfer the cells to low serum, serum-free medium, or mouse serum prior to the injection.

2. The cells should be resuspended in approximately 100 μl of PBS per site of injection. Use a 25-gauge needle.

3. Hold the mouse as shown in Figure 6.7. Inject under the skin.

NOTES

i. Normal doses of mammalian cells will be between 10^5 and 10^7 cells/injection.

ii. Because of potential infections of the mouse colony, injections of live viruses, bacteria, or fungi normally are not recommended. These antigens are commonly killed or inactivated prior to injection.

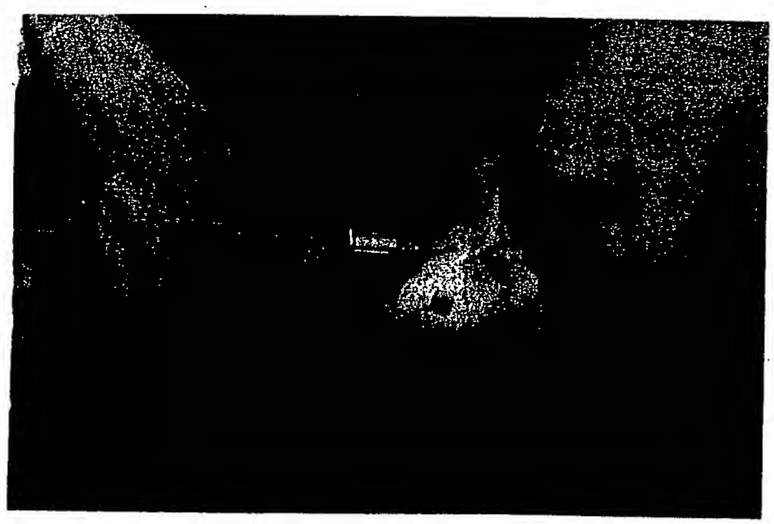


FIGURE 6.7
Subcutaneous injection of a mouse.

SUBCUTANEOUS IMPLANTS—NITROCELLULOSE

Protein antigens immobilized on nitrocellulose often make exceptionally good immunogens. This is probably due to their slow release from the paper, thus behaving somewhat like an adjuvant (p. 96). Not all antigens show increased immunogenicity using this methodology, but some do. The antigen is bound to paper and is implanted on the back of the mouse's neck, a location that makes it difficult for the mouse to disturb the surgical clip.

1. Incubate a solution of the antigen (no more than 1.0 mg/ml in PBS) with a sheet of nitrocellulose (0.1 ml/cm^2) at room temperature for 1 hr in a humid atmosphere.

2. Wash the sheet three times with PBS.

3. Anesthetize the mouse by injecting a suitable drug (see Chapter 5, p. 95). For mice, 0.05 ml of Nembutal ip (sodium pentobarbitone, 40–85 mg/kg) is appropriate. The mouse will be ready for the operation in about 1–2 min.

4. Swab the skin on the mouse's back, just below the base of the neck, with alcohol. Raise the skin with a forceps and make a 1.5-cm incision with sterile scissors. Pull the skin from the inner body wall and insert a 0.5-cm^2 piece of the nitrocellulose. Close the incision with a skin clamp and return the mouse to its cage.

5. Take weekly serum samples beginning about 14 days after the implant. About 10 days after the serum titer drops, the final boost can be given. The final boost will still be an iv injection.

INTRAVENOUS INJECTIONS

Intravenous injections (iv) can be used for two purposes. When immunizing mice, their main use will be to deliver the final boost just before a hybridoma fusion (p. 210). However, iv injections also are useful to ensure that the antigen is seen by the immune system. A rapid and strong response can be expected, as the antigen will be collected quickly in the spleen, liver, and lungs. The antigen will be processed quickly and no continued release of the antigen into the immune system can be expected. Consequently, these types of injections produce a short-lived response.

Intravenous injections should never be used as primary injections, and they must not contain large particulate matter. Because the injection will introduce the antigen directly into vital organs, harsh chemicals must be avoided. Similarly, adjuvants such as Freund's should not be used (p. 96).

1. Isolate the mouse in a small cage or container. Heat the mouse with an infrared lamp. This will increase the blood supply to the tail, making the veins easier to inject. Be careful of the length of time the mouse is left under the lamp. If it's too hot for your hand, it's too hot for the mouse.

2. Move the mouse to a restraining device as shown in Figure 6.8.

3. Swab a portion of the tail with alcohol about 1.5 cm below the base. The veins on the tail should be easily visible.

4. Use a 1.0-ml syringe fitted with a 26- or 27-gauge needle. Hold the tail firmly with one hand and guide the needle into one of the veins. Gently draw back on the plunger. If the needle is in the vein, there will be very little resistance and blood will appear in the barrel. If there is strong resistance and no blood appears, the needle is not in the vein. Withdraw the needle and move to a second site or to another vein. This technique may require a little practice before you can hit the vein readily. Practice with injections of PBS.

5. After you are sure the needle is in the vein, slowly deliver the injection. Pause a few seconds, remove the needle, and return the mouse to its cage. The antigen should be in solution and no adjuvant should be included. Except for specialized injections, the maximum amount to give a mouse by this method is 0.2 ml.

NOTES

i. Injection by this route into immunized animals may cause an anaphylactic reaction. This can be prevented by a prior injection of an antihistamine. Contact your local animal committee for guidance.

ii. Solutions for iv injections should not contain high concentrations of denaturing agents and should be free of toxic chemicals such as sodium azide.

FIGURE 6.8
Intravenous injection of a mouse.

INJECTIONS DIRECTLY INTO LYMPHOID ORGANS

These are injections for more specialized delivery of antigens. They are often appropriate for small amounts of antigen and particularly for secondary or later boosts. In theory, these types of injections may be the best methods for giving a final boost, but because they demand more skill, they are not in common use. The two most frequently used sites of injection are the footpad and the spleen.

In general, footpad and spleen injections should be done only for highly specialized antigens and then only after consulting local authorities for the proper protocols.

Identifying Individual Mice

Beginning with the first test bleeds, it is essential to mark the mice so that the immune response can be monitored in individuals. There are a number of methods that are currently used to identify mice. These include ear punches, toe clips, and tail markings. If your animal facility has a standard method, consult them for the proper codes. If not, an acceptable method that is not harmful to the mice is to color the toes of their hindlegs with an indelible marker. This procedure is relatively easy, and, because the marks are on the back legs, the mice don't seem to work as hard to remove the markings as on other sites. Even so, the marks need to be reapplied twice a week.

If there is a large difference between the responses in individual mice, it may be worthwhile to isolate individual mice in separate cages to ensure that the proper mouse is given the final boost.

Test Bleeds

Except in unusual circumstances, it is seldom worthwhile to fuse antibody-secreting cells from animals that do not have a usable titer of antibodies in their serum. Periodic test bleeds collected from immunized animals should be checked for the desired antibodies. Tests are run conveniently on small batches of serum prepared from tail bleeds of immunized mice.

The test bleed will yield small samples of polyclonal sera. These sera should be tested in assays that will detect the presence of antibodies specific for the antigen. These tests are discussed in detail in Chapters 10–14.

To make appropriate comparisons in these tests, two practical matters need to be considered. First, the test bleeds should always be titered to monitor the development of the response. The appropriate dilutions will depend on the strength of the response and on the type of assay, but in general 1 in 5 or 1 in 10 dilutions will be satisfactory. Second, the proper negative control should be another polyclonal serum and not an unrelated monoclonal antibody. Most often, this negative control will be serum collected either from another uninjected animal or from an animal that has been boosted with an unrelated antigen. Although it is not always necessary, using serum from a test bleed collected before immunization of the animal is the best negative control. These bleeds are known as preimmune sera.

COLLECTING SERA FROM A MOUSE BY TAIL BLEED

1. Isolate the mouse in a small cage or container. Heat the mouse under an infrared lamp for a few minutes. This treatment will increase the blood flow to the tail, but be careful not to hurt the mouse. If it's too hot for your hand, then it's too hot for the mouse. Place the mouse in a restricted space as shown in Figure 6.8.

2. Swab a portion of the tail about 1.5–2 inches from the body with alcohol. Using a sterile scalpel, nick the underside of the tail across one of the lower veins that should be visible. Collect several drops of blood in a tube and return the mouse to its cage.

3. Incubate the blood at 37°C for 1 hr. Flick the tube several times to dislodge the blood clot.

4. Transfer to 4°C for 2 hr or overnight.

5. Spin at 10,000*g* for 10 min at 4°C.

6. Remove the serum from the cell pellet. Discard the cell pellet and spin the supernatant a second time for 10 min. Remove the serum, being careful to avoid the packed cell pellet.

7. Add sodium azide to 0.02% and test. Any remaining serum may be frozen at −20°C. The yield is approximately 100–200 μl.

Deciding to Boost Again or to Fuse

Three factors will influence the decision to proceed with the production of monoclonal antibodies. They all are related to the quality and strength of the immune response. First is whether the antibodies recognize the antigen of interest. This is the most straightforward of the factors and the simplest to determine. The second is a complicated set of properties of the antibodies themselves and the strength of the immune response. These properties are manifested as different titers of antibodies and different affinities of the antibody for the antigen. The third factor is the appearance of spurious antibody activities against unrelated antigens.

In many cases the tests will be relatively easy, and the interpretation apparent. First, the sera should be checked for antibodies that bind to the immunogen itself. For example, if a purified antigen is used, sera could easily be tested for activity in a simple antibody capture assay (p. 175), or if whole cells are used then testing for binding to the cell surface should be done first (p. 184). However, if the monoclonal antibodies will be used for tests other than these simple assays, test bleeds should be checked in assays that resemble, as closely as possible, the tests for which the antibodies are being prepared. For many antibodies, the most useful test will be the immunoprecipitation of the antigen (p. 429). This assay is easy when only testing a few samples, and it will identify antibodies that will be useful in a large number of tests that depend on binding to the native antigen. If, however, the antibodies will be used extensively in immunoblot analyses, in immunohistochemical staining, or in other tests in which many antibodies may fail to work, these tests should be run as well.

Second, sera should also be monitored for the concentration of specific antibodies by titering the test bleeds in the appropriate assays. As the immune response matures, higher levels of specific antibodies will be found. However, higher levels of antibodies do not necessarily mean higher affinities. If high affinity is crucial to the intended use of the antibody, the sera should be titered and compared in assays that are sensitive to antibody affinity, such as immunoprecipitation.

The third factor to consider is the appearance of antibody activities against extraneous antigens. This response may be directed against other antigens in your preparations or may be a response to other antigens in the mouse's environment, including invasion by a pathogenic organism. If the mouse is ill, do not proceed with hybridoma construction. Isolate the mouse in a separate cage and allow it to recover before continuing. If a particularly valuable antigen is being used, more care and veterinary help may be needed. If the antibody activities are to contaminating antigens in the immunogen, a decision must be made whether to proceed. In general, making monoclonal antibodies against complex and multicomponent antigens is a very useful way of isolating specific immunochemical probes, particularly when the antigen is difficult to purify further. However, if the response against the other antigens continues to increase without a concomitant strengthening of the response to the desired antigen,

other approaches may need to be taken. Either other mice should be tested (other individuals or other strains) or the antigen may need to be purified further before proceeding.

DEVELOPING THE SCREENING METHOD

Because most hybridoma cells grow at approximately the same rate, the tissue culture supernatants from all the fusion wells usually are ready to screen within a few days of one another. This means that screening is normally the most labor-intensive segment of hybridoma production. Care in developing the proper screen will help to keep the amount of work needed to identify positive wells to a minimum.

Approximately 1 week after the fusion, colonies of hybrid cells will be ready to screen. During the screening, samples of tissue culture media are removed from wells that have growing hybridomas and are tested for the presence of the desired antibodies. Successful fusions will produce between 200 and 20,000 hybridoma colonies, with 500–1000 colonies being the norm. Depending on the fusion, individual wells will become ready to screen over a 2- to 6-day period. Typically, the first wells would be ready to screen on day 7 or 8, and most of the wells will need to be screened within the next 4 or 5 days.

A good screening procedure must: (1) reduce the number of cultures that must be maintained to a reasonable level (seldom more than 50 cultures at one time), (2) identify potential positives in 48 hr or less (24 hr or less is ideal), and (3) be easy enough to perform for all the needed wells. Positive wells may be as rare as 1 in 500 or as common as 9 out of 10. Several screening steps can be combined to identify the desired clones, as long as the first screen reduces the tissue culture work to a manageable level. After the first round of screens, handling the tissue culture necessary for 100 wells is difficult for one person, 50 wells is reasonable, and less than 20 is relatively simple.

> All screening procedures must be tested and validated before the fusion has begun. After the fusion, there is seldom enough time to try out new ideas or to refine methods. The test bleeds should be used to set up and test the screening assay.

Screening Strategies

There are three classes of screening methods, antibody capture assays, antigen capture assays, and functional screens. Currently, most screens are done by either antigen or antibody capture, but as functional assays become easier to use, more fusions will be screened by these methods. Table 6.5 depicts several of the more common screening techniques.

In general, the more antigens in the immunizing injections, the more difficult the screen. Researchers with pure or partially pure antigens should use methods for antibody capture. If the subcellular location of an antigen is known, positive tissue culture supernatants can be identified by cell staining. If the immunizations used complex antigen solutions, procedures such as immunoprecipitation or other antigen capture assays may be the only alternatives.

In addition to the tests described below, any of the assays used for analyzing antigens can be adapted for use as a screen (see Chapters 10–14).

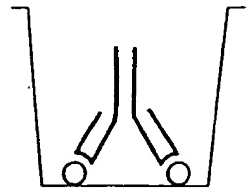
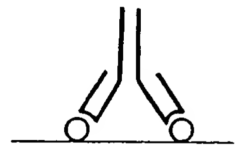
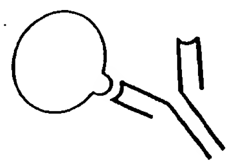
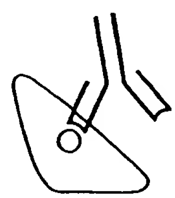
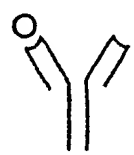
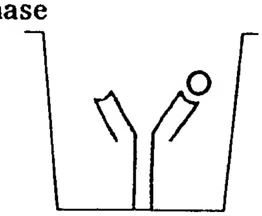
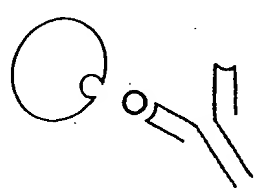
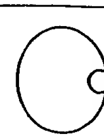
Antibody Capture Assays

Antibody capture assays are often the easiest and most convenient of the screening methods. In an antibody capture assay the following sequence takes place: the antigen is bound to a solid substrate, the antibodies in the hybridoma tissue culture supernatant are allowed to bind to the antigen, the unbound antibodies are removed by washing, and then the bound antibodies are detected by a secondary reagent that specifically recognizes the antibody. In this assay the detection method identifies the presence of the antibody, thus determining a positive reaction.

Most antibody capture assays rely on an indirect method of detecting the antibody. This is commonly done with a secondary reagent such as rabbit anti-mouse immunoglobulin antibodies. These antibodies can be purchased from commercial suppliers or can be prepared by injecting purified mouse immunogloblins into rabbits (see p. 622). The rabbit antibodies can be purified, labeled with an easily detectable tag (pp. 288 and 319), and used to determine the presence of mouse monoclonal antibodies.

Alternatively, positives can be located by other reagents that will bind specifically to antibodies. Two proteins that may be used for these reactions are protein A and protein G (see p. 615). Both of these polypeptides are bacterial cell wall proteins that have high affinities for a site in the Fc portion of some antibodies. Protein A and protein G can be purified and labeled with an appropriate tag.

TABLE 6.5
Methods for Screening Hybridoma Fusions

Method	Examples
Antibody Capture	PVC wells  Nitrocellulose  Whole cells  Permeabilized cells 
Antigen Capture	Ab/Ag in solution  Ab/Ag on solid phase 
Functional	Blocking  Depletion 

Advantages	Disadvantages
Easy Rapid	Need pure or partially pure antigen Doesn't discriminate between high- and low-affinity Ab
Relatively easy Doesn't need pure Ag Learn Ag locale	Ag prep can be tedious Doesn't discriminate between high- and low-affinity Ab
Only detect high-affinity Ab	Unless you have pure labeled Ag, assay is tedious and slow
Rapid	Need pure labeled Ag Setting up solid phase is tricky
Ab immediately useful	False positives Potentially tedious
Ab immediately useful Only detect high affinity Ab	Tedious Ag must be limiting

ANTIBODY CAPTURE ON NITROCELLULOSE—DOT BLOTS*

If the antigen is a protein that is available in large amounts, dot blots are one of the assays of choice. The antigen is bound directly to the nitrocellulose sheet. Many assays are performed on a single sheet; therefore, the manipulations are simple.

Assays using polyvinylchloride multiwell plates in place of nitrocellulose sheets are good alternatives to dot blots. They are discussed on pp. 180 or 182.

1. A protein solution of at least 1 μg/ml is added to a nitrocellulose sheet at 0.1 ml/cm². Allow the protein to bind to the paper for 1 hr. Higher concentrations of proteins will increase the signal and make screening faster and easier. If the amount of protein is not limiting, concentrations of 10–50 μg/ml should be used. Nitrocellulose can bind approximately 100 μg of protein per cm².

2. Wash the nitrocellulose sheet three times in PBS.

3. Place the sheet in a solution of 3% BSA in PBS with 0.02% sodium azide for 2 hr to overnight. To store the sheet, wash twice in PBS and place at 4°C with 0.02% sodium azide. For long-term storage, shake off excessive moisture from the sheet, cover in plastic wrap, and store at −70°C.

4. Place the wet sheet on a piece of parafilm, and rule with a soft lead pencil in 3-mm squares. Cut off enough paper for the number of assays.

5. Apply 1 μl of the hybridoma tissue culture supernatant to each square. Incubate the nitrocellulose sheet on the parafilm at room temperature in a humid atmosphere for 30 min.

   Along with dilutions of normal mouse serum, include dilutions of the mouse serum from the last test bleed as controls. Dilutions of the test sera are essential to control correctly for the strength of the positive signals. Mouse sera will often contain numerous antibodies to different regions of the antigen and therefore will give a stronger signal than a monoclonal antibody. Therefore, dilutions need to be used to lower the signal. Good monoclonal antibodies will appear 10-fold less potent than good polyclonal sera.

6. Quickly wash the sheet three times with PBS, then wash two times for 5 min each with PBS.

*Adapted from Sharon et al. (1979); Glenney et al. (1982); Hawkes et al. (1982); Herbrink et al. (1982); Huet et al. (1982); Yen and Webster (1982); and reviewed in Hawkes (1986).

7. Add 50,000 cpm of ^{125}I-labeled rabbit anti-mouse immunoglobulin per 3-mm square in 3% BSA/PBS with 0.02% sodium azide (about 2.0 ml/cm^2).

8. After 30–60 min of incubation with shaking at room temperature, wash extensively with PBS until counts in the wash buffer approach background levels.

9. Cover in plastic wrap and expose to X-ray film with a screen at −70°C.

NOTES

i. The types of bonds that hold protein to nitrocellulose are not known. However, the binding is blocked by oils or other proteins. Wear gloves and use virgin nitrocellulose sheets. For short- or long-term storage, do not leave the paper in buffers containing other proteins. Slow exchange occurs and this will lower the strength of the signal. For long-term storage, freeze the paper in plastic wrap at −70°C.

ii. In all of the antibody capture assays, the method for detecting the antibody can be substituted with other techniques. Most techniques employ either ^{125}I-labeled rabbit anti-mouse immunoglobulins or horseradish peroxidase-labeled rabbit anti-mouse immunoglobulins. For nitrocellulose tests using enzyme-linked reagents, only substrates that yield insoluble products should be used (p. 681). Both enzyme- and ^{125}I-labeled reagents can be purchased from commercial suppliers or prepared in the lab (p. 319). If the detection method uses horseradish peroxidase rather than ^{125}I-labeled rabbit anti-mouse immunoglobulin, sodium azide will block the development of the color.

iii. In all the assays in which proteins are bound to nitrocellulose or polyvinylchloride, the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use whatever works best for you. If the blocking solutions are prepared in advance and stored, sodium azide should be added to 0.02% to prevent bacterial or fungal contamination.

ANTIBODY CAPTURE IN POLYVINYLCHLORIDE WELLS—^{125}I DETECTION*

Antibody capture assays in polyvinylchloride (PVC) plates are one of the most commonly used assays. Each well serves as a separate assay chamber, but because they are molded together the manipulations are simple. Two variations of these techniques are listed here, one for ^{125}I detection and one for enzyme-linked assays (p. 182). Both are easy and accurate. The radioimmune assay is easier to quantitate, but the enzyme assay is adequate for most purposes and avoids the problems of radioactive handling.

1. Prepare a solution of approximately 2 μg/ml of the antigen in 10 mM sodium phosphate (pH 7.0). Higher concentrations will speed the binding of antigen to the PVC, but the capacity of the plastic is only about 300 ng/cm^2, so the extra protein will not bind to the PVC. However, if higher concentrations are used, the protein solution can be saved and used again. Many workers prefer to use 50 mM carbonate buffer (pH 9.0) in place of the phosphate buffer.

2. Add 50 μl of antigen to each well. Incubate at room temperature for 2 hr or overnight at 4°C.

3. Remove the contents of the well. If the solution will not be saved, it can be removed by aspiration or by flicking the liquid into a suitable waste container.

4. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient.

5. Fill the wells with 3% BSA/PBS with 0.02% sodium azide. Incubate for at least 2 hr at room temperature.

6. Wash the plate twice with PBS.

7. Add 50 μl of the tissue culture supernatant. Incubate 1 hr at room temperature.

8. Wash the plate twice with PBS.

9. Add 50 μl of 3% BSA/PBS containing 50,000 cpm of ^{125}I-labeled rabbit anti-mouse immunoglobulin to each well. Incubate 1 hr at room temperature. (^{125}I-Labeled reagents can be purchased or prepared as described on p. 324.)

*Adapted from Catt and Tregear (1967); Salmon et al. (1969).

10. Wash the plate with PBS until there are no more counts in the wash buffer.

11. Either cut the wells apart and count in a gamma counter or expose the entire plate to film.

NOTES

i. In all the assays in which proteins are bound to nitrocellulose or polyvinylchloride, the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use those that work best for you. If the blocking solutions are prepared in advance and stored, sodium azide should be added to 0.02% to prevent bacterial or fungal contamination.

ii. Antigen-coated plates can be prepared in advance and stored. After the blocking solution has been removed, store at −70°C.

*ANTIBODY CAPTURE IN POLYVINYLCHLORIDE WELLS—ENZYME-LINKED DETECTION**

As an alternative to using ^{125}I-labeled reagents for antibody capture assays in polyvinylchloride (PVC) plates (p. 180), enzyme-linked assays can be employed. The assay is performed identically to the ^{125}I-labeled assay except the detection methods are changed.

1. Prepare a solution of approximately 2 μg/ml of the antigen in 10 mM sodium phosphate (pH 7.0). Higher concentrations will speed the binding of antigen to the PVC, but the capacity of the plastic is only about 300 ng/cm^2, so the extra protein will not bind to the PVC. However, if higher concentrations are used, the protein solution can be saved and used again. Many workers prefer to use 50 mM carbonate buffer (pH 9.0) in place of the phosphate buffer.

2. Add 50 μl of antigen to each well. Incubate at room temperature for 2 hr or overnight at 4°C.

3. Remove the contents of the well. If the solution will not be saved, it can be removed by aspiration or by flicking the liquid into a suitable waste container.

4. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient.

5. Fill the wells with 3% BSA/PBS (no sodium azide). Incubate for at least 2 hr at room temperature.

6. Wash the plate twice with PBS.

7. Add 50 μl of the tissue culture supernatant. Incubate 1 hr at room temperature.

8. Wash the plate twice with PBS.

9. Add 50 μl of 3% BSA/PBS (without sodium azide) containing a dilution of horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin antibody to each well. Incubate 1 hr at room temperature. (Horseradish peroxidase-labeled reagents can be purchased or prepared as described on p. 344. Most commercial reagents should be diluted 1 in 1000 to 1 in 5000. Try several dilutions in preliminary tests and choose the best.)

10. Wash the plate with PBS three times.

*Adapted from Catt and Tregear (1967); Salmon et al. (1969); Engvall and Perlmann (1972).

11. During the final washes prepare the TMB substrate solution. Dissolve 0.1 mg of 3′,3′,5′,5′-tetramethylbenzidine (TMB) in 0.1 ml of dimethylsulfoxide. Add 9.9 ml of 0.1 M sodium acetate (pH 6.0). Filter through Whatman No. 1 or equivalent. Add hydrogen peroxide to a final concentration of 0.01%. This is sufficient for two 96-well plates. (Hydrogen peroxide is normally supplied as a 30% solution. After opening, it should be stored at 4°C, where it is stable for 1 month.)

12. After the final wash in PBS, add 50 μl of the substrate solution to each microtiter well.

13. Incubate for 10–30 min at room temperature. Positives appear pale blue.

14. Add 50 μl of stop solution, 1 M H_2SO_4, to every well. Positives now appear bright yellow. To quantitate the binding, read the results at 450 nm.

NOTES

i. In all the assays in which proteins are bound to polyvinylchloride, the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use whatever works best for you.

ii. Antigen-coated plates can be prepared in advance and stored. After the blocking solution has been removed, store at −70°C.

iii. Do not include sodium azide in solutions when horseradish peroxidase is used for detection.

ANTIBODY CAPTURE ON WHOLE CELLS—CELL-SURFACE BINDING*

If the subcellular location of an antigen is known, cell staining assays can be used to screen hybridoma tissue culture supernatants. Two assays are given here, one for cell-surface screening using detection with ^{125}I-labeled reagents and a second for internal localization using enzyme-labeled reagents. Also, any of the techniques for cell staining in Chapter 10 can be adapted for screening.

1. Prepare a suspension of target cells at $1\text{–}2 \times 10^6$ cells/ml in 1% BSA/PBS with 0.1% sodium azide. If the cell pellets are particularly difficult to see, add a drop of a suspension of red blood cells or other colored particle that will not interfere with the assay.

2. Add 100 μl of the cell suspension to the wells of a V-bottomed polyvinylchloride (PVC) plate.

3. Centrifuge the PVC plate for 5 min at 400*g*. Many centrifuge manufacturers supply suitable plate carriers for these types of assays.

4. Carefully remove the supernatants by aspiration.

5. Resuspend the cell pellet without adding any buffer by tapping the plate or by using a microshaker. Dispersing the cell pellet is important for the rapid binding of antibody to the surface antigens.

6. Add 50 μl of tissue culture supernatant. Incubate at 4°C for 1 hr with periodic shaking.

7. Centrifuge the PVC plate for 5 min at 400*g*. Remove the tissue culture supernatant by aspiration.

8. Wash the cells twice by resuspending in 200 μl of ice cold 1% BSA/PBS with 0.1% sodium azide and centrifuging the plate.

9. Add 50 μl of ice-cold 1% BSA/PBS with 0.1% sodium azide containing 50,000 cpm of ^{125}I-labeled Fab fragment of rabbit anti-mouse immunoglobulin antibodies. Incubate at 4°C for 90 min with periodic shaking. If the target cells do not have a receptor for the Fc portion of immunoglobulins, then ^{125}I-labeled rabbit anti-mouse immunoglobulin antibody may be used in place of the Fab fragment.

*Goldstein et al. (1973); Jensenius and Williams (1974); Morris and Williams (1975); Welsh et al. (1975); Galfre et al. (1977); Williams (1977).

10. Centrifuge the PVC plate as before. Wash the cells three times with ice-cold 1% BSA/PBS with 0.1% sodium azide.

11. Cut the wells and count in a gamma counter or expose the plate to film.

NOTES

i. Keeping the cells cold and in the presence of sodium azide throughout this procedure will slow the rate of capping and internalization of the surface antigens.

ii. In all of the antibody capture assays, the method for detecting the antibody can be substituted with other techniques. Most techniques employ either ^{125}I-labeled rabbit anti-mouse immunoglobulin antibodies or enzyme-linked rabbit anti-mouse immunoglobulin antibodies. Both these reagents can be purchased from commercial suppliers or prepared in the lab (p. 321).

ANTIBODY CAPTURE ON PERMEABILIZED CELLS—CELL STAINING*

One major advantage of using cell staining in hybridoma screens is that the assays give an extra level of information. Unlike other antibody capture assays that rely on the simple detection of antibody, cell staining also determines the localization. This extra information makes cell staining a good assay when using complex antigens.

Both fluorochrome- and enzyme-labeled reagents can be used to detect the presence of the antibodies (see Chapter 10 for more details), but if the levels are high enough to be detected using enzyme-labeled reagents, enzyme methods should be used. Enzyme-labeled assays can be scored by using the light microscope. Scoring assays using the fluorescent microscope will give more resolution, but long-term observation under this microscope is disorienting for most people.

1. Grow cells in standard tissue culture conditions on standard tissue culture plates with fetal calf serum. Some staining will be more pronounced on subconfluent cells, some on fully confluent cells.

2. Pour off the medium, and flood the plate with PBS.

3. Pour off the PBS. Flood the plate with freshly prepared 50 : 50 acetone/methanol mixture. Incubate at room temperature for 5 min.

4. Pour off the acetone/methanol, and allow to air dry (approximately 5 min).

5. Score the bottom of the plate with a marking pen to form a grid of small squares to identify the location of the hybridoma tissue culture supernatants. Fifty tests can easily be done on one 100-mm tissue culture dish.

6. Add 2–5 μl of tissue culture supernatant to the fixed and permeabilized cell sheet above the appropriate mark. Incubate for 1 hr at room temperature in a humid atmosphere.

7. Wash the entire plate by flooding with PBS. Pour off the PBS and repeat three times.

8. For a 100-mm dish, add 3 ml of rabbit anti-mouse immunoglobulin antibody-horseradish peroxidase solution (diluted 1/200 in 3% BSA/PBS) to the plate. Incubate for 1 hr at room temperature.

9. Pour off and wash three times with PBS.

*Lane and Lane (1981); see also Chapter 10 for historical and alternative methods.

10. During the last wash, dissolve 6 mg of 3,3′-diaminobenzidine in 9 ml of 50 mM Tris (pH 7.6). A small precipitate may form. Add 1 ml of 0.3% (wt/vol) $NiCl_2$ or $CoCl_2$. Filter through Whatman No. 1 filter paper (or equivalent). This is sufficient for one 100-mm plate.

11. Add 10 μl of 30% H_2O_2. (H_2O_2 is generally supplied as a 30% solution and should be stored at 4°C, where it will last about 1 month.)

12. Add 10 ml of substrate solution per 100-mm dish. Develop at room temperature with agitation until the spots are suitably dark. A typical incubation would be approximately 1–15 min.

13. Pour off the enzyme substrate and wash several times with water. Store in water with 0.02% sodium azide.

14. Look for black/brown spots in the marked squares and examine positives under the microscope.

NOTE

i. Cells grown in calf serum often show high background staining due to binding of the second antibody to calf immunoglobulins.

Antigen Capture Assays

In an antigen capture assay, the detection method identifies the presence of the antigen. Often this is done by labeling the antigen directly. These assays require the monoclonal antibody to have a high affinity for antigen since the labeled antigen is normally added at very low concentration in free solution.

There are two types of antigen capture assays, and these assays differ by the order in which the steps are performed. In one variation, the antibodies in the tissue culture supernatant are bound to a solid phase first, and then the antigen is allowed to react with the antibody. In the second variation, the antibody–antigen complex is allowed to form prior to the binding of the antibody to a solid phase. In either case, once the antibody–antigen complexes are bound to the solid support, the unbound antigen is removed by washing, and positives are identified by detecting the antigen.

Detection of the antigen can be performed by a number of techniques. If the antigen is available in pure form, it can be labeled by radiolabeling, fluorescent tagging, or enzyme coupling. If the antigen itself is an enzyme, positives may be determined by the presence of the enzymatic activity. Any property that is unique to the antigen can be used to identify positives.

ANTIGEN CAPTURE ON NITROCELLULOSE— REVERSE DOT BLOT*

Reverse dot blot assays are more complicated to use than many of the other screening assays, but they are particularly valuable if pure or partially pure antigen is available, although only in limited quantities. The monoclonal antibodies in the supernatants are "captured" on an anti-immunoglobulin antibody layer, previously bound to nitrocellulose or PVC (p. 192 for this variation), and then labeled antigen is added. Positives can be determined by the location of the antigen. Because the antigen can be labeled to high specific activity with either ^{125}I or enzyme, very little antigen is used in the screening procedure. However, the assays are tricky to set up and demand careful use.

1. Prior to the assay, purify the immunoglobulin fraction from rabbit anti-mouse sera using one of the standard methods (p. 288). Purification on protein A beads is probably the easiest for the rabbit antibodies. Alternatively, purchase the purified antibodies from a commercial source.

2. Cut nitrocellulose paper to the size of a dot blot apparatus. Add rabbit anti-mouse immunoglobulin solution (approximately 200 μg of purified antibody/ml in PBS) to nitrocellulose paper. Use 10 ml/100 cm^2. Incubate for 60 min at room temperature.

3. Wash the paper three times with PBS, 5 min for each wash.

4. Incubate in 3% BSA/PBS with 0.02% sodium azide for 1 hr at room temperature.

5. Load into a 96-well dot blot apparatus.

6. Add 50 μl of hybridoma tissue culture supernatant to each well. Incubate for 1 hr at room temperature.

7. Draw the supernatant through the nitrocellulose paper by applying vacuum to the bottom chamber of the dot blot apparatus.

8. Wash the nitrocellulose paper and wells three times with 3% BSA/PBS.

9. Remove the paper from apparatus and incubate with ^{125}I-labeled antigen (10 ml/96-well sheet, 50,000 cpm/well in 3% BSA/PBS) at room temperature for 1 hr with shaking. (^{125}I-Labeled antigens can be prepared as described on p. 324.)

*Adapted from Wide and Porath (1966); Catt and Tregear (1967); Engvall and Perlmann (1971); Van Weeman and Schuurs (1971).

10. Wash the paper with PBS until counts in the wash buffer approach background levels.

11. Cover in plastic wrap and expose to X-ray film at −70°C with a screen.

NOTES

i. The types of bonds that hold protein to nitrocellulose are not known. However, the binding is blocked by oils or other proteins. Wear gloves and use virgin nitrocellulose sheets. For short- or long-term storage, do not leave the paper in buffers containing other proteins. Slow exchange occurs and this will lower the strength of the signal. For long-term storage, freeze the paper in plastic wrap at −70°C.

ii. In all the assays in which proteins are bound to nitrocellulose, the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use whatever works best. If the blocking solutions are prepared in advance and stored, sodium azide should be added to 0.02% to prevent bacterial or fungal contamination.

ANTIGEN CAPTURE IN POLYVINYLCHLORIDE WELLS*

This assay is similar to the nitrocellulose reverse dot blot assay described on p. 190. The major difference is the use of polyvinylchloride (PVC) plates in place of nitrocellulose. This makes the handling of the individual assays easier, because each well is used for a separate assay; with the nitrocellulose, this is achieved by using a dot blot apparatus. The major disadvantage of using the PVC is the lower binding capacity of the PVC wells.

1. Add 50 μl of affinity-purified rabbit anti-mouse immunoglobulin in PBS (20 μg/ml) to each well. Incubate for 2 hr at room temperature or overnight at 4°C. Many workers prefer to use 50 mM carbonate buffer (pH 9.0) in place of the PBS.

   Because of the low binding capacity of PVC, the signal will be stronger with affinity-purified rabbit anti-mouse immunoglobulin antibodies. These are the subset of antibodies in the anti-mouse immunoglobulin sera that bind to mouse antibodies. Affinity-purified antibodies can be prepared in the laboratory (p. 313) or can be purchased from commercial sources. When using concentrations above about 20 μg/ml, the solution should be saved for reuse.

2. Wash twice with PBS.

3. Add 200 μl of 3% BSA/PBS with 0.02% sodium azide to each well. Incubate for at least 2 hr at room temperature.

4. Wash twice with PBS. Add 50 μl of tissue culture supernatant to each well. Incubate for 2 hr at room temperature.

5. Wash three times with PBS. Add 50 μl of 3% BSA/PBS with 0.02% sodium azide containing 50,000 cpm of ^{125}I-labeled antigen per well. Incubate for 1 hr at room temperature. (Procedures for labeling antigens are described on p. 324.)

6. Wash with PBS until counts in the wash buffer approach background levels.

7. Either cut the wells apart and count in a gamma counter or expose the plate to X-ray film at −70°C with a screen.

*Adapted from Wide and Porath (1966); Catt and Tregear (1967); Engvall and Perlmann (1971); Van Weemen and Schuurs (1971).

NOTE

i. In all the assays in which proteins are bound to polyvinylchloride the remaining binding sites on the solid support must be blocked by adding extraneous proteins. Solutions that are commonly used include 3% BSA/PBS, 1% BSA/PBS, 0.5% casein/PBS, 20% skim dry milk/PBS, or 1% gelatin/PBS. There are conflicting reports in the literature as to the best reagents to use, and this may reflect differences in suppliers or slight differences in techniques. Test several and use whatever works best for you. If the blocking solutions are prepared in advance and stored, sodium azide should be added to 0.02% to prevent bacterial or fungal contamination.

ANTIGEN CAPTURE IN SOLUTION—IMMUNOPRECIPITATION

Immunoprecipitation is seldom used for screening hybridoma fusions, because the assays are tedious and time consuming. However, because the antigen is normally detected after SDS-polyacrylamide electrophoresis, it is simple to discriminate potential positives from authentic ones. The added information gained about the molecular weight of an antigen makes these assays particularly useful when using complex antigens.

1. Prepare sufficient radiolabeled antigen for overnight detection of 100 samples. Samples can be labeled directly using ^{125}I (p. 324) or prepared from radiolabeled extracts of cells (p. 429).

2. Either use tissue culture supernatants directly or pool in such a way that no more than 98 samples need to be handled (p. 215).

3. Label microfuge tubes and add samples of radiolabeled antigen to each. Add 50 μl of hybridoma tissue culture supernatants to each tube. Include one positive control (probably from the last test bleed) and one negative control (probably from a nonimmune mouse). Incubate for 1 hr on ice.

   If all classes of immunoglobulins are wanted, 30 min into the incubation add 0.5 μl of rabbit anti-mouse immunoglobulin serum. Normally it is easiest to add this as 10 μl of 1 in 20 dilution of the serum in PBS. Keep on ice.

4. Add 20 μl of a 10% suspension of prewashed SAC (p. 620). Incubate for 30 min on ice.

5. Spin for 1 min at 10,000g. Remove supernatant by aspiration. Resuspend the pellet in 750 μl of PBS.

6. Spin for 1 min at 10,000g and repeat wash.

7. Spin for 1 min at 10,000g and remove supernatant by aspiration. Add 50 μl of Laemmli sample buffer (p. 684). To make the resuspension easier, snap freeze by placing the tubes in a dry ice–ethanol bath. Resuspend and load onto a polyacrylamide gel. Handle as for normal electrophoresis (p. 636).

Functional Assays

In functional assays, the antibodies in the hybridoma tissue culture supernatants are used either to block a reaction or as a molecular handle to deplete an essential component of a reaction mix. Any antibodies that are identified using these assays form an immediately useful set of reagents. However, the assays are difficult to perform and interpret, and therefore are seldom used.

PRODUCING HYBRIDOMAS

Although hybridoma production is the most discussed of the stages of monoclonal antibody preparation, most of the steps have been analyzed in enough detail that they are now routine. The ease with which this stage proceeds is dependent on how well the previous stages of immunization and development of the screen have gone. A strong immune response and the use of a good screening method will make the production of the hybridomas an easier task.

Once a good immune response has developed in an animal and an appropriate screening procedure has been developed, the construction of hybridomas is ready to begin. For the actual fusion, antibody-secreting cells are isolated from the appropriate lymphoid tissue, mixed with myeloma cells, centrifuged to generate good cell-to-cell contacts, and fused with polyethylene glycol (PEG). The fused cells are then removed from the PEG solution, diluted into selective medium, and plated in multiwell tissue culture dishes. Beginning approximately 1 week later, samples of the tissue culture supernatants are removed from wells that contain growing hybridomas and tested for the presence of the appropriate antibodies. Cells from positive wells are grown, single-cell cloned, and frozen. Finally, the monoclonal antibodies are collected and used.

Hybridoma production demands good tissue culture facilities and a worker with tissue culture experience. An experienced worker will be able to perform the entire fusion procedure from removal of the lymphoid tissue to the plating of the final fused cells in less than 2 hr. Little work is then required until the screening begins in about 1 week. This step is the most labor intensive of the entire project. Approximately 1 week is needed to complete the screening of the hybridoma wells, and if the fusion has been successful, another 2 weeks of tissue culture work will be needed until a suitable stage for a break has been reached. *Do not begin hybridoma production without the time needed for these operations.*

Although resultant hybridomas are relatively easy to grow, in the first stages following the fusion, they may be particularly fragile and need extra care. Because they are the final result of a long series of operations, and because they are produced as individual clones with no backup, the cells are quite valuable. At the early stages contaminated cultures cannot be recovered.

Chapter 7 (p. 245) contains descriptions of the techniques used for growing and maintaining hybridoma and myeloma cell lines as well as lists of appropriate growth media.

Preparation for Fusions

Prior to the time of fusion, several solutions must be prepared. In addition, unless you have purchased batches of fetal bovine serum and PEG that have been prescreened by manufacturers for their use in fusions, these solutions should be tested.

SCREENING FOR GOOD BATCHES OF FETAL BOVINE SERUM

Only about one in five lots of fetal bovine serum (FBS) is particularly good at supporting hybridoma growth. The key constituents that distinguish good batches of serum from bad are not known. Order test batches from several suppliers or purchase prescreened serum directly from the distributor.

1. Test each batch of FBS against your present lot. Test the FBS with your most commonly used myeloma line as well as two hybridoma lines (if available). If possible, use one hybridoma that is easy to maintain and one that is more difficult.

2. For each lot of serum to test, prepare 30 ml of 10% FBS in medium (p. 247) without any further additives. Dispense 100 μl of the test medium in all the wells of a 96-well tissue culture dish using a multiwell pipettor. Prepare three trays per test and place the trays back in a CO_2 incubator to adjust the pH.

3. Wash the three test cell lines (one myeloma and two hybridomas) in medium without serum. The cells should be healthy and growing rapidly before the test. Resuspend the cells in medium without serum at a concentration of approximately 10^5 cells/ml. You will need approximately 2.5 ml for these tests.

4. Add 100 μl of the cell suspensions to each of the eight wells in the left-hand row of the test plates—one cell line per plate; three plates/sample of FBS.

5. Using an eight-well multipipettor mix the contents of the left hand row. Then remove 100 μl from the first row and do serial 1 in 2 dilutions across the plate. Incubate at 37°C in a CO_2 incubator.

6. Check the wells under a microscope after 7, 10, and 14 days. The wells in the left-hand side of the plate should all grow. Depending on the ability of the individual batches of serum to support growth, you will see growth extending to the wells with smaller number of cells. This assay tests directly for the ability of different serum samples to replace the feeder effects of high-density hybridoma culture and mimics the problems of individual cells attempting to grow out from either single-cell cloning or hybrid fusions.

NOTES

i. Good batches of serum should support growth of as few as 20 cells per well. Do not purchase batches that support less than 100.

ii. Any method that is used for single-cell cloning can be adapted to screen serum batches.

iii. Serum is stable when stored at −20°C for 1 year.

PREPARING OPI

OPI is a solution of oxaloacetate, pyruvate, and insulin that helps support the growth of hybridoma and myeloma cells at low densities. It is not required for high-density culture.

1. To prepare 100 ml of 100× OPI, dissolve 1.5 grams of oxaloacetate, 500 mg of sodium pyruvate in 100 ml of H_2O suitable for tissue culture work.

2. Add 2000 IU (international units) of bovine insulin.

3. Filter-sterilize.

4. Dispense in sterile tubes in 2.0-ml aliquots. Freeze at −20°C.

   OPI is stable at −20°C for 6 months to 1 yr.

PREPARING POLYETHYLENE GLYCOL

1. Melt PEG 1500 in a 50°C water bath. Place a small glass vial on a top-loading balance, and add melted PEG. Add either 0.5 gram or 0.3 gram of PEG, depending on which fusion method will be used (pp. 211 or 212). Most workers use PEG 1500 for fusions, but others use anything from PEG 1000 to PEG 6000 with good results.

2. Cap the vials and autoclave to sterilize.

   PEG is stable at room temperature for many years.

NOTE

i. PEG can also be weighed dry and then autoclaved.

SCREENING FOR GOOD BATCHES OF POLYETHYLENE GLYCOL

Because fusions require so little polyethylene glycol (PEG), good batches will last a long time. Because differences in batches normally are small, and only the odd batch is unusable, most workers do not bother to test different lots of PEG. Bad batches contain trace amounts of toxic chemicals. Buy the highest grade of PEG that is available.

1. Add 100 μl of medium with 10% serum to each well of a 96-well microtiter dish (one plate per batch of PEG to be tested).

2. Wash myeloma cells by centrifugation at 400*g* for 5 min (approximately 10^6 cells per assay). Resuspend the cells in medium without serum and then respin.

3. While washing the myeloma cells, melt one vial (0.5 gram) of each of the PEG samples to be tested at 50°C (p. 201 for PEG preparation). Add 0.5 ml of medium without serum to each vial and place in a 37°C water bath.

4. Resuspend the cells in medium without serum and aliquot samples containing 10^6 cells into fresh centrifuge tubes (one tube per batch of PEG). Spin at 800*g* for 5 min. Carefully remove the supernatant from the cell pellet.

5. Resuspend each cell pellet with the 50% PEG solutions by pipetting. Include one control that is resuspended in medium without serum or PEG. Incubate at room temperature for 2 min. Add 10 ml of medium with 10% FBS. Take 100 μl of this suspension and dilute into a second 10 ml of medium with 10% FBS. Spin the final dilution at 400*g* for 5 min.

6. Aspirate the supernatant and resuspend the myeloma cells in 1.0 ml of medium with serum. Transfer 100 μl of the cell suspension into each of the eight wells on the left-hand side of the 96-well tissue culture dish. Using an eight-well multipipettor do 1 in 2 serial dilutions across the plate. Return to the CO_2 incubator.

7. Check the plates at day 14. Good batches of PEG will only slightly inhibit growth and will resemble the no PEG controls. Other batches should be discarded.

NOTE

i. The American Type Culture Collection (ATCC) and Boehringer Mannheim supply high-quality PEG that does not need to be tested prior to use.

Drug Selections

Hybridoma cell lines are selected by the addition of drugs that block the de novo synthesis of nucleotides (see p. 277 for details of the theory of drug selection). The most commonly used agents are aminopterin, methotrexate, and azaserine. All are effective agents to select against the growth of the myeloma fusion partner. When using aminopterin or methotrexate, de novo purine and pyrimidine synthesis are blocked, whereas azaserine blocks only purine biosynthesis. Consequently, aminopterin and methotrexate are supplemented with hypoxanthine and thymidine. Azaserine solutions are supplemented with hypoxanthine.

*PREPARING HAT SELECTION MEDIUM**

Hypoxanthine, aminopterin, and thymidine selection (HAT) medium is commonly prepared from two stock solutions, 100× HT and 100× A.

1. To prepare 100 ml of 100× HT, dissolve 136 mg of hypoxanthine and 38 mg of thymidine in 100 ml of H_2O suitable for tissue culture. Heat gently (70°C) if they do not dissolve completely. The 100× stock is 10 mM hypoxanthine and 1.6 mM thymidine.

2. To prepare 100 ml of 100× A, add 1.76 mg of aminopterin to 100 ml of H_2O suitable for tissue culture. Add 0.5 ml of 1 N NaOH to dissolve. Titrate with 1 N HCl to neutral pH, being sure not to overshoot to acid pH, as aminopterin is sensitive to acid pH. The concentration of the 100× stock is 0.04 mM aminopterin.

3. Filter-sterilize the two solutions independently.

4. Dispense 2.0-ml aliquots in sterile tubes. Store at −20°C.

   Both 100× HT and 100× A are stable at −20°C for 1 year.

*Littlefield (1964).

PREPARING HMT SELECTION MEDIUM

HMT selection medium is commonly prepared from two stock solutions, 100× HT and 100× M.

1. To prepare 100 ml of 100× HT, dissolve 136 mg of hypoxanthine and 38 mg of thymidine in 100 ml of H_2O suitable for tissue culture. Heat gently (70°C) if they do not dissolve completely. The 100× stock is 10 mM hypoxanthine and 1.6 mM thymidine.

2. To prepare 100 ml of 100× M, add 49 mg of methotrexate to 100 ml of H_2O suitable for tissue culture. Add 0.5 ml of 1 N NaOH to dissolve. Titrate with 1 N HCl to neutral pH. The 100× stock solution is 1 mM methotrexate.

3. Sterilize the 100× HT and 100× M solutions by filtration.

4. Dispense 2.0-ml aliquots in sterile tubes. Store at −20°C.

   Both 100× HT and 100× M are stable at −20°C for 1 year.

*PREPARING AH SELECTION MEDIUM**

1. To prepare 100 ml of 100× AH, add 0.136 gram of hypoxanthine in H_2O suitable for tissue culture. Heat to 70°C to dissolve, and then add 10 mg of azaserine. The 100× stock is 0.58 mM azaserine and 10 mM hypoxanthine. 100× H will be needed for growing hybridoma cells while removing the azaserine selection. 100× H is prepared as above, but without the addition of azaserine.

2. Filter sterilize.

3. Dispense into sterile tube in 2.0-ml aliquots. Store at −20°C.

   100× AH is stable when stored at −20°C for 1 year.

*Foung et al. (1982).

Expanding and Freezing Positive Clones

After a positive well has been identified, the cells are transferred from the culture in the 96-well plate to 0.5 ml of medium supplemented with 20% fetal bovine serum, 1× OPI, and 1× AH in a 24-well plate. After the 24-well culture becomes dense, it is transferred into 5.0 ml in a 60-mm dish and then to 10 ml in a 100-mm dish. Once the cells are transferred into the 60-mm dish, the drug selection can begin to be removed. This is done by first growing the cells for several passages in complete medium with hypoxanthine but lacking azaserine, or in complete medium with hypoxanthine and thymidine but lacking aminopterin or methotrexate. In either case, growing the cells with the base but without the drug allows all of the inhibitors to be diluted to a safe level before removing the bases.

At the 100-mm dish stage, the cells should be frozen. This is a convenient stage to collect 10 ml of supernatant, if any further testing of hybridomas needs to be done before concentrating on particular clones. However, if the correct clones have already been identified, the cells should be single-cell cloned as early as possible. This can be begun as early as at the 60-mm dish stage. Techniques for the freezing and storage of hybridoma cell lines are described on p. 257.

Often the transfer of hybridomas from one size of culture dish to the next is a difficult step to maintain cell viability. Presumably this is caused by the dilution of the growth factors in the medium and may be caused in part by overgrowth in the previous stage. If these problems exist, try using feeder cultures at these stages (see pp. 220–221 for the preparation of feeder cultures). Also, adding a sample of the diluted culture back into the original well will serve as a good backup if any problems arise.

COMMENTS ■ More Fusions?

After the screen has been completed, the decision on the appropriate next steps will depend on the number of positives that have been identified. If no positives are found, and the immunization yielded a strong response, the fusion should be repeated, but the choice of screening method should be reevaluated. If the immune response was weak, new approaches to the immunization should be tried. If only a few positive clones were identified, these should be tested as early as possible to determine whether they will perform adequately in the appropriate assays. If a comprehensive set of immunochemical reagents are needed, additional fusions are likely to be needed. If the fusion has been very successful (greater than 50 positives), it is seldom worthwhile and often practically impossible to carry and maintain all the clones. In these situations many of clones are likely to result from fusion of sibling antibody-secreting cells and therefore will not generate new antibody activities. Some sort of secondary screen should be considered to identify particularly valuable clones. This might be based on affinity or perhaps subclass of the resultant antibodies.

Single-cell Cloning

After a positive tissue culture supernatant has been identified, the next step is to clone the antibody-producing cell. The original positive well will often contain more than one clone of hybridoma cells, and many hybrid cells have an unstable assortment of chromosomes. Both of these problems may lead to the desired cells being outgrown by cells that are not producing the antibody of interest. Single-cell cloning ensures that cells that produce the antibody of interest are truly monoclonal and that the secretion of this antibody can be stably maintained.

Isolating a stable clone of hybridoma cells that all secrete the correct antibody is the most time-consuming step in the production of hybridomas. Depending on the chances of the original positive being derived from a single cell, the easiest and quickest methods to prepare single-cell clones will differ. If the positive well contains multiple clones or if secretion of the antibody is highly unstable, the cloning should be done in two or more stages. In the first cloning, you should try to identify a positive well with only a few clones, and then try to isolate a single-cell clone from this stage. This often can be achieved by a combination of different cloning methods. For example, quick cloning by limiting dilution could be followed by cloning with a single cell pick.

Because hybridoma cells have a very low plating efficiency, single-cell cloning is normally done in the presence of feeder cells or conditioned medium. Good feeder cells should secrete the appropriate growth factors and should have properties that allow them to be selected against during the future growth of the hybridomas. Feeder cell cultures are normally prepared from splenocytes, macrophages, thymocytes, or fibroblasts.

> To ensure that a hybridoma is stable and single-cell cloned, continue repeating the cloning until every well tested is positive.

PREPARING SPLENOCYTE FEEDER CELL CULTURES

Although splenocyte feeders can be used immediately, they are most effective when they are prepared approximately 1 day before the single-cell cloning. Because spleen cells do not grow in normal tissue culture conditions, they are lost during the subsequent expansion of the hybridoma cells. Use a female mouse of the same genetic background as your hybridoma.

1. Sacrifice the mouse. See your local authorities on animal handling for advice on the proper humane procedures. Remove the spleen aseptically from the mouse and place in a 100-mm tissue culture dish containing 5 ml of medium without serum (see p. 209). Trim off and discard any contaminating tissue from the spleen.

2. Tease apart the spleen using 19-gauge needles on 1.0-ml syringes. Continue to tease until most of the cells have been released and the spleen has been torn into very fine parts. Disrupt any cell clumps by pipetting. Transfer the cells and medium into a conical centrifuge tube leaving behind all of the larger pieces of tissue. Wash these clumps and the plate with an additional 5 ml of medium without serum and combine with the first 5 ml.

3. Allow the cell suspension to sit at room temperature for approximately 2 min. This will allow the larger cell clumps to settle to the bottom of the tube. Carefully remove the medium and cells avoiding the sediment, and transfer to 100 ml of medium with 10% fetal bovine serum prescreened to support hybridoma growth (1 spleen per 100 ml is about 10^8 cells/100 ml or 10^6/ml). Either use directly or prepare conditioned medium.

4. **Either:** To use directly for 96-well cloning (pp. 222–224), plate 50 μl of the spleen cell solution into each of the wells of a 96-well tissue culture dish. Allow to grow for 24 hr at 37°C.

   Or: To use directly for soft-agar cloning (p. 226), the medium with the feeder cells is used to dilute the hybridoma cell suspension prior to mixing with the soft-agar.

   Or: To prepare conditioned medium, transfer the splenocyte cell suspension to several tissue culture dishes. Place at 37°C in a CO_2 incubator for 3 days. Collect the cell suspension and remove the cells by centrifugation at 400*g* for 10 min. Filter sterilize and dispense in convenient sizes. Freeze at −70°C. Use the conditioned medium mixed 1:1 with medium supplemented with 20% FBS and 2× OPI.

NOTE

i. To avoid any possible problems with a particular spleen feeder culture, it may be best to combine several batches.

PREPARING FIBROBLAST FEEDER CELL CULTURES

Certain fibroblast cultures secrete the necessary factors to allow the growth of hybridoma cells at low plating densities. Early studies used fibroblast cultures that had been treated with mitomycin C or lethal doses of irradiation. Both of these treatments made it impossible for the feeder cells to contaminate future cultures of the hybridomas. More recently, this has been shown not to be necessary for fibroblast cultures that adhere strongly to the plastic tissue culture surface. Other studies have compared the ability of different fibroblast cells to support single-cell cultures of hyridoma cells and have found that the human diploid cells MRC 5 are the most effective in this test. These cells are not an established cell line, and so will need to be replaced in the future by another source. The MRC 5 cells are currently available from several sources including the American Type Culture Collection.

1. The MRC 5 cells are grown and maintained in Ham's F12 medium supplemented with 10% fetal bovine serum. They should be used at passages below 40.

2. Trypsinize the cells and count. Prepare a solution of 2×10^5 cells/ml of medium with 10% fetal bovine serum prescreened to support hybridoma growth.

3. For cloning using 96-well tissue culture dishes (pp. 222–224), add 50 μl of the cell suspension to the wells. Allow the cultures to grow for 1 day at 37°C.

   Or: For soft-agar cloning using 60- or 100-mm tissue culture dishes (p. 226), add 10 ml of the cell suspension to a 100-mm dish or 3 ml to a 60-mm dish. Allow the cells to adhere to the plastic overnight at 37°C. Remove the medium and add the soft agar hybridoma cell suspension to the plate.

SINGLE-CELL CLONING BY LIMITING DILUTION

Cloning hybridoma cells by limiting dilution is the easiest of the single-cell cloning techniques. Two approaches are given below, one rapid technique for generating cultures that are close to being single-cell cloned and one for single-cell cloning directly.

Even though every attempt is made to ensure that the cells are in a single-cell suspension prior to plating, there is no way to guarantee that the colonies do not arise from two cells that were stuck together. Therefore, limiting dilution cloning should be done at least twice to generate a clonal population.

Limiting Dilution (Rapid)

1. Using a multiwell pipettor (8-, 12-, or 96-well), add 50 μl of medium with 20% FBS and 2× OPI to each well of a 96-well plate. The wells should already contain 50 μl of feeder cells (pp. 220 or 221) giving 100 μl total volume/well.

2. The hybridomas should be growing rapidly. Remove 100 μl of the hybridoma cell suspension using a pipetman and transfer to the top left-hand well. Mix by pipetting.

3. Do 1 in 2 doubling dilutions down the left-hand row of the plate (8 wells, 7 dilution steps). Discard tip.

4. Do 1 in 2 doubling dilutions across the plate using an 8-well multi-pipetter.

5. Clones should be visible by microscopy after a few days and normally will be ready to screen after 7–10 days. Score the wells by microscopy. There should be a line running on a 45° diagonal that contains approximately the same number of clones per well. If the cells are nearly cloned when you start, screen only wells with one or two clones. If not, screen a selection of wells with multiple clones as well as all those with only one clone.

6. Select the best wells and either grow up or repeat the cloning procedure directly.

Limiting Dilution (Slow)

1. The hybridomas should be healthy and rapidly growing at the time of cloning. Prepare four dilution tubes with medium supplemented with 20% fetal bovine serum and 2× OPI for each cell to be cloned. Three tubes should have 2.7 ml and the fourth should have 3.0 ml.

2. Add 10 μl of the hybridoma cells to the tube containing the 3.0 ml of medium. Do 1 in 10 dilutions of the hybridomas by removing 0.3 ml and transferring into the 2.7-ml tubes.

3. Add 100 μl of each dilution into 24 of the wells of a 96-well tissue culture plate (24 wells/dilution; 4 dilutions/plate, i.e., one hybridoma/plate). The wells should already contain 50 μl of feeder cells (pp. 220 or 221), giving 150 μl total volume/well. If the cells from the highest dilution are plated first, then the pipet does not need to be changed during the plating.

   If many hybridomas are being cloned at the same time, it may be worthwhile to plate the dilutions by using a 10-ml or larger pipet. One drop from these pipets will deliver approximately 100 μl.

4. Clones will begin to appear in 4 days and should be ready to screen starting about days 7–10.

   Screens can be done from wells containing multiple clones as well as from wells containing only single clones.

*SINGLE-CELL CLONING BY PICKS**

Cloning hybridomas by picking a single cell from a growing culture is the only cloning method that ensures that clones arise from a single cell. During the cloning procedure, the cell is followed under the microscope to be certain that the clone comes from only one cell.

1. Add approximately 100 μl of medium with 20% FBS and 2× OPI to the wells of a 96-well plate (approximately 20 wells/hybrid). The wells should already contain 50 μl of feeder cells (pp. 220 or 221) giving 150 μl total volume/well.

2. At the time of the cloning all cells should be growing rapidly. Do serial 1 in 5 dilutions of the hybridoma cells in 60-mm dishes. Use about 0.3 ml into 1.2 ml; this will allow enough volume to cover the bottom of the plate, but not so deep as to make the pipetting difficult. Observe the cells under the microscope and choose a plate with well-separated cells.

3. Use a drawn out 50-μl capillary pipet connected to a mouth pipetting device with a 0.2-μm filter fitted in the line. Partially fill the pipet with complete medium from a separate plate without cells. While watching under the microscope, draw a single cell into the pipet. Move to an area of the plate without any cells and blow out the cell to make sure you have only one cell. Draw it up again and transfer to one of the wells with feeders. With practice, single-cell picks take about 1 min.

4. The clones should be ready to screen in 7–10 days.

*J. Wyke (pers. comm.).

NOTE

i. Because this technique demands working under the microscope on the open bench, one might expect contamination to be common. However, the only portion of the tissue culture medium that is exposed to the open air for long is the dish that you are picking from and you only transfer a very small volume at one time. So the chances of contamination are low. Needless to say, this technique should only be done in an area without drafts.

SINGLE-CELL CLONING BY GROWTH IN SOFT AGAR

Cloning of hybridoma cells in semisolid medium is one of the most commonly used methods for producing single-cell clones. The technique is easy, but, because it is performed in two stages, it does take longer than other methods. Not all cells will grow in soft agar, and there may be a bias on the type of colony that appears. However, most of the commonly used myeloma fusion partners have relatively good cloning efficiencies in soft agar, and consequently, so do most hybridomas.

Even though every attempt is made to ensure that the cells are in a single-cell suspension prior to plating, there is no way to guarantee that the colonies do not arise from two cells that were stuck together. Therefore, single-cell cloning in soft agar should be repeated at least twice before the cells are considered clonal.

1. Prior to cloning prepare 3% agarose (Seaprep 15/45, FML Corporation or equivalent) in H_2O suitable for tissue culture. Sterilize by autoclaving. This is stable for 6 months to 1 year.

   Prepare double-strength medium, normally from powdered medium. Add 100 μg/ml of gentamicin, and sterilize by filtration. Store at 4°C. Stable for about 1 month at 4°C, but at that time if fresh glutamine is added to 2 mM the shelf life can be extended to 3 months.

2. Melt agarose in a boiling water bath or in a microwave oven and cool to 37°C.

3. To the 2× medium add fetal bovine serum to 20% and OPI to 2×. Warm to 37°C in a water bath.

4. Cells should be healthy and growing rapidly at the time of cloning. The cells should be as free of clumps as possible. Do 1 in 10 dilutions of hybridomas in 1× medium. If not using feeders, the 1× medium is prepared by diluting a sample of the complete 2× medium with sterile H_2O. If using feeders that grow in suspension, the medium used for these dilutions should be the cell suspension from the feeder cell preparation (p. 220). If using fibroblast feeders (p. 221), these cells should be plated on the tissue culture dishes to be used for the cloning 24 hr earlier, and the 1× medium should be prepared by diluting the 2× complete medium.

5. Add 150 μl of cells from the dilutions between 10^5 and 10^2 cells/ml to 60-mm tissue culture plates (2 plates/dilution). Do not bother to count cells. If you are uncertain about the exact concentration of cells, it is easier to do an extra dilution than to count the cells.

6. Mix the 3% agarose and the 2× medium 1 : 1. Add 4 ml to each plate, and mix by pipetting.

7. Place the plates at 4°C for 45 min and then transfer to 37°C in a CO_2 incubator.

8. Macroscopic clones will appear beginning about day 10. Pick clones from the highest dilution that shows growth. Remove a plug of agarose containing the colony with a sterile Pasteur pipet. Transfer the plug to 1 ml of medium in a 24-well plate. Disperse the clone by pipetting.

9. Supernatants from these wells will normally be ready for screening 48–72 hr later.

NOTE

i. As an alternative, the cells may be grown in the dilution tubes themselves (Civin and Banquerigo 1983). Add 2 ml of the 1.5% agarose/medium solution to each tube and grow as described above.

Unstable Lines

If hybridomas continue to produce less than 100% positive wells, even after four or more single-cell cloning steps, the lines probably have an unstable assortment of chromosomes. If the antibodies produced by these cells are particularly valuable, extra work to save these lines may be necessary. Two strategies are used. In the first and most straightforward, the single-cell cloning is continued on a regular basis, trying to isolate a stable subclone. Perhaps surprisingly, this often works. The screening assays should be adjusted to screen not only for the presence of the appropriate antibody, but also for the levels of antibody produced. Wells that contain a stable subclone of the original should produce higher levels of antibodies. If the stable variant is generated early in the proliferation within a well, the differences in antibody production between the well containing the variant and those that do not will be significant. At this stage many workers stop screening with an antigen-specific assay and only screen for the level of mouse antibody produced (see p. 560 for examples). After a stable line is generated, the specificity of the antibody should be reestablished.

A second strategy is to refuse the important line with a myeloma and allow the chromosomes to reassort from the beginning, hoping to isolate the stable variant from this source. To date, most re-fusions have been done by standard techniques and extensive screening. However, the introduction of a selectable drug selection marker into a suitable myeloma cell line should make selection against the parental myelomas easier. The hybridoma would carry a functional HPRT gene, while the myeloma would carry, for example, a neomycin gene. Selection for both genes should yield only successful secondary hybridomas.

Contamination

During the early stages of the fusion, contamination will mean the loss of the well or the fusion; however in later stages, important hybridomas can sometimes be saved.

CONTAMINATION IN THE FUSION WELLS— A FEW WELLS ONLY

1. Contaminated wells can be identified by their unusual pH or turbidity. Confirm the presence of the contaminating organisms by observing under the microscope. Mark the wells.

2. Move to the tissue culture hood and carefully remove the lid. If the underside of the lid is damp, replace with a new lid. Dry the top and edges of the plate itself by aspiration before replacing. If there is contaminated medium on the lid, autoclave the whole plate without any further work.

3. Remove the medium from the contaminated well by aspiration. Try to avoid generating any aerosols. Add enough 10% bleach to the well to bring the level right to the rim. Allow it to sit for 2 min at room temperature.

4. Remove the bleach from the contaminated well by aspiration. Add enough ethanol to the well to bring the level right to the rim. Remove by aspiration and repeat.

5. Dry the well by aspiration.

CONTAMINATION IN THE FUSION WELLS—GROSS

1. Autoclave the plates.

CONTAMINATION OF A CLONED LINE

1. If the line has been frozen, it is easiest to go back to the most recent freeze down and thaw a fresh vial of the cells.

2. If the line has not been frozen, inject the cells into mice that have been primed for ascites production (p. 274). The animals must be of a compatible genetic background to your hybrids (e.g., BALB/c × BALB/c into BALB/c or BALB/c × C57Bl/B6 into BALB/c × C57B1/B6 F_1). If no mice have been primed with 0.5 ml of pristane the required 1 week in advance, inject 0.5 ml of Freund's adjuvant into the peritoneum. Wait 4 hr to 1 day and inject the hybridomas. Inject at least two mice for each contaminated culture.

3. When and if ascites develop, tap the fluid and transfer into a sterile centrifuge tube (see p. 274 for more information on ascites production).

4. Spin the ascites at 400g for 5 min at room temperature.

5. Remove the supernatant. Resuspend the cell pellet in 10 ml of medium supplemented with 10% fetal bovine serum and transfer to a tissue culture plate. The supernatant can be checked for production of the appropriate antibody. If positive, save for use.

6. Handle as for normal hybridomas, except keep the cells separate from the other cultures until there is little chance of the contamination reappearing.

The success rate may be as high as 80%.

NOTE

i. Animals injected with infected cultures should be kept isolated from the main animal colony.

Classing and Subclassing of Monoclonal Antibodies

Many techniques for using monoclonal antibodies require antibodies with specific properties. One set of these properties is unique to the individual antibody itself and includes such variables as specificity and affinity for the antigen. These properties all depend on differences in the antigen-combining domain of the antibody and can be assayed by comparing the properties of the monoclonal antibodies in tests that measure antigen binding activity.

A second set of important properties for monoclonal antibodies is determined by the structure of the remainder of the antibody, sequences encoded by the antibody common regions. These properties include the class or subclass of the heavy chain or the light chain. The different classes or subclasses will determine the affinity for important secondary reagents such as protein A (see p. 616). The type of heavy and light chain can be distinguished by simple immunochemical assays that measure the presence of the individual light- and heavy-chain polypeptides. This is normally achieved by raising antibodies specific for the different mouse heavy- and light-chain polypeptides (p. 622). The production of these antibodies is possible because the light- and heavy-chain polypeptides from different species are sufficiently different to allow them to be recognized as foreign antigens. Most often these anti-mouse immunoglobulin antibodies are raised in rabbits as polyclonal sera, and then the antibodies specific for a particular heavy or light chain are purified on immunoaffinity and immunodepletion columns. Although these chain-specific rabbit anti-mouse immunoglobulin antibodies can be made in the laboratory, it is normally easier to purchase them from commercial sources. There are a large number of different assays used, and some of the more common are listed below.

*DETERMINING THE CLASS AND SUBCLASS OF A MONOCLONAL ANTIBODY BY OUCHTERLONY DOUBLE-DIFFUSION ASSAYS**

Originally, the Ouchterlony double-diffusion assays were the most common method for determining class and subclass of a monoclonal antibody. They have been largely superseded by other techniques, but they still are useful, particularly when only a few assays will be performed. In these assays, samples of tissue culture supernatants (often concentrated tenfold) are pipetted into a well in a bed of agar. Class- and subclass-specific antisera are placed in other wells at equal distance from the test antibody. The two groups of antibodies diffuse into the agar. As they meet, immune complexes form, yielding increasing larger complexes as more antibodies combine. When large multimeric complexes form, the immune complexes will precipitate, forming a line of proteins that is either visible to the naked eye or that can be stained to increase the sensitivity. The precipitated proteins form what is referred to as a precipitin line.

1. Prepare a 10-ml sample of tissue culture supernatant from a hybridoma. Grow the cells in medium supplemented with 10% FBS and allow the culture to overgrow and die.

2. Spin the tissue culture supernatant at 1000*g* for 10 min. Collect the supernatant. If the supernatant is not clear of all debris, either filter it through a 0.45-μm filter (sterility is not important) or spin at 7000*g* for 15 min.

3. Concentrate the supernatant 10-fold using an ultrafiltration manifold. This is most easily done with adaptors that are designed to concentrate in the centrifuge. Many of the ultrafiltration specialty companies now supply these devices; follow the manufacturers' instructions. Remove the tissue culture supernatant when the 10 ml sample has been reduced to 1 ml.

   Tissue culture supernatants may also be concentrated by ammonium sulfate precipitation (p. 298).

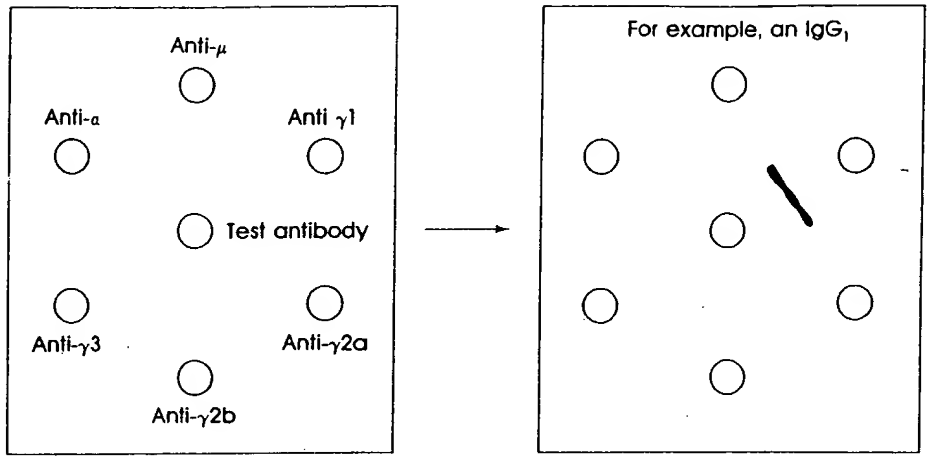
4. Prepare a 1.4% agarose solution in PBS with 5 mM EDTA. Melt the agarose in a boiling water bath or in a microwave. Cool to 45°C.

   Ouchterlony plates can also be purchased commercially.

5. On a level surface pipet 3 ml of the agarose solution onto the top of a 3 × 5-cm clean glass slide. The agarose should form a layer about 2 mM deep. The surface tension of the agarose should hold the agarose on the slide. Allow to harden at room temperature.

*Ouchterlony (1949).

6. Using a 200-μl capillary pipet or a commercial apparatus, carefully core vertical small holes in the agarose in a pattern that looks like this:



If the capillary pipet is attached to a pipetting device, a light suction while preparing the wells will allow the plugs to be withdrawn easily.

7. Add 5 μl of rabbit anti-mouse immunoglobulin sera specific for the various classes, subclasses, or light chains to each of the wells in the outer ring.

8. Add 5 μl of the concentrated tissue culture supernatant to the middle well.

9. Incubate in a humid atmosphere overnight at room temperature.

10. Score positive reactions by the appearance of a precipitin line between the wells with reactive antibodies.

NOTE

i. The sensitivity of these assays can be increased by staining the bands with Coomassie brilliant blue. Cover the gel with wet filter paper and place in a 50°C oven. Incubate until dry. Wet the paper and remove from the gel. Wash for 30 min in several changes of PBS. Repeat the drying procedure. Stain with Coomassie for 15 min (p. 649). Destain in 7% acetic acid, 25% methanol.

DETERMINING THE CLASS AND SUBCLASS OF MONOCLONAL ANTIBODIES USING ANTIBODY CAPTURE ON ANTIGEN-COATED PLATES

Any of the assays used to screen hybridoma fusions that detect antibodies with a secondary anti-mouse immunoglobulin antibody can be adapted to screen for class or subclass. For example, if the detection method used ^{125}I-labeled rabbit anti-mouse immunoglobulin to locate antibodies bound to the antigen, then substituting anti-class or subclass-specific antibodies for the ^{125}I-reagent will identify the type of heavy chains. An example of these types of reactions is given below using an antigen bound to 96-well PVC plates, but similar tests could be developed for any of the antibody capture assays.

1. Prepare a solution of approximately 2 μg/ml of the antigen in 10 mM sodium phosphate (pH 7.0). Higher concentrations will speed the binding of antigen to the PVC, but the capacity of the plastic is only about 300 ng/cm^2, so the extra protein will not bind to the PVC. However, if higher concentrations are used, the protein solution can be saved and used again. Many workers prefer to use 50 mM carbonate buffer (pH 9.0) in place of the phosphate buffer.

2. Add 50 μl of antigen to each well. Incubate at room temperature for 2 hr or overnight at 4°C.

3. Remove the contents of the well. If the solution will not be saved, it can be removed by aspiration or by flicking the liquid into a suitable waste container.

4. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient.

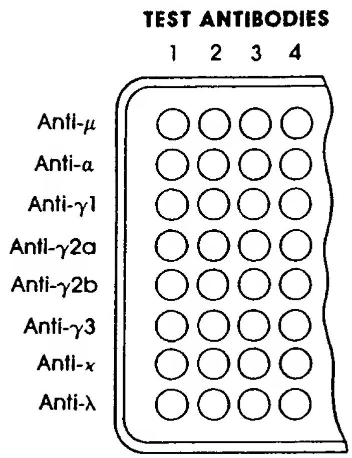
5. Fill the wells with 3% BSA/PBS (no sodium azide). Incubate for at least 2 hr at room temperature.

6. Wash the plate twice with PBS.

7. Add 50 μl of each tissue culture supernatant to be tested to every well of a vertical row (8 wells/test). Incubate 1 hr at room temperature.

8. Wash the plate twice with PBS.

9. Add 50 μl of 3% BSA/PBS (without sodium azide) containing a dilution of horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin class- or subclass-specific antibody to each well as shown below:



Incubate 1 hr at room temperature. (Horseradish peroxidase-labeled reagents can be purchased or prepared as described on p. 344. Most commercial reagents should be diluted 1 in 1000 to 1 in 5000. Try several dilutions in preliminary tests and choose the best.)

10. Wash the plate with PBS three times.

11. During the final washes prepare the TMB substrate solution. Dissolve 0.1 mg of 3',3',5',5'-tetramethylbenzidine (TMB) in 0.1 ml of dimethylsulfoxide. Add 9.9 ml of 0.1 M sodium acetate (pH 6.0). Filter through Whatman No. 1 or equivalent. Add hydrogen peroxide to a final concentration of 0.01%. This is sufficient for two 96-well plates. (Hydrogen peroxide is normally supplied as a 30% solution. After opening, it should be stored at 4°C, where it is stable for 1 month.)

12. After the final wash in PBS, add 50 μl of the substrate solution to each microtiter well.

13. Incubate for 10–30 min at room temperature. Positives appear pale blue.

14. Add 50 μl of stop solution, 1 M H_2SO_4, to every well. Positives now appear bright yellow. To quantitate the binding, read the results at 450 nm.

NOTE

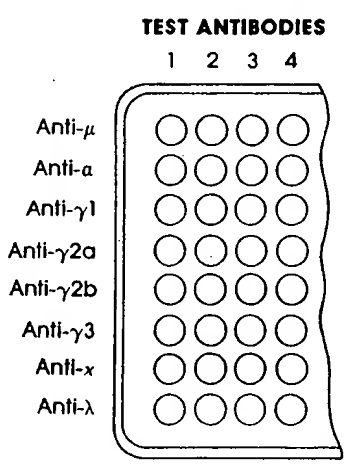
i. Do not include sodium azide in solutions when horseradish peroxidase is used for detection.

DETERMINING THE CLASS AND SUBCLASS OF MONOCLONAL ANTIBODIES USING ANTIBODY CAPTURE ON ANTI-Ig ANTIBODIES

One of the easiest methods for determining the class and subclass of a monoclonal antibody is to bind class- or subclass-specific antibodies to the wells of a polyvinylchloride (PVC) plate. The test monoclonal antibody is added to each well, but will bind only to wells coated with antibodies that are specific for its subclass or class. These bound antibodies are detected using a secondary antibody specific for all mouse antibodies.

1. Purify the antibodies from rabbit anti-mouse immunoglobulin class- or subclass-specific antibodies. Techniques for these purifications are discussed in Chapter 8. For most purposes, protein A beads are probably the easiest to use. (Rabbit anti-mouse immunoglobulin class- and subclass-specific sera can be purchased from several suppliers.)

2. After purification dilute the antibodies to 20 μg/ml in PBS. Add 50 μl to the wells of a PVC plate in the pattern below. Each monoclonal antibody being tested will need one row.



3. Incubate for 2 hr to overnight at room temperature in a humid atmostphere.

4. Remove the antibodies and save for future use. The antibodies can be reused approximately five times.

5. Fill the wells with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr or overnight at room temperature.

6. Wash three times with PBS. Add 50 μl of tissue culture supernatant from each hybridoma to the appropriate wells.

7. Incubate at room temperature for 2 hr in a humid atmosphere. Shake out the unbound antibody, and wash three times with PBS.

8. Add 50,000 cpm of ^{125}I-labeled rabbit anti-mouse immunoglobulin antibody to each well (diluted in 3% BSA/PBS with 0.02% sodium azide).

9. Incubate for 2 hr at room temperature in a humid atmosphere. Discard the iodinated antibodies in an appropriate waste container.

10. Wash the wells three times with PBS. Cut the wells from the plate and count in a gamma-counter.

NOTE

i. Other detection methods can be substituted for the iodinated antibodies. Common alternatives include enzyme-labeled reagents.

Selecting Class-switch Variants

During the normal development of a humoral response, the predominant class of antibodies that are produced changes, beginning primarily with IgMs and developing into IgGs. These changes and others like them occur by genetic rearrangements that move the coding region for the antigen binding site from just upstream of the IgM-specific region to the IgG region. These events are described in detail in Chapter 2 (p. 7). These rearrangements help the host animal tailor the immune response to the various types of infection. The different classes and subclasses of antibodies also have properties that make them more or less useful in various immunochemical techniques. These differences make the preparation of antibodies of certain classes or subclasses very valuable.

Recently, it has been shown that a process that appears similar to the natural class and subclass switching occurs in vitro, although at a very low frequency. Therefore, any population of hybridomas will have a small proportion of cells secreting antibodies with a different class or subclass of antibody. The antigen binding site will be identical in these antibodies. If these cells can be identified and cloned, then antibodies with the same antigen binding site but with different class or subclass properties can be isolated. These "shift variants" generally are useful in one of two cases, either switching from IgM to IgG or from IgG_1 to IgG_{2a}. Often these switches are used to produce antibodies that bind with higher affinity to protein A.

When trying to identify any class or subclass switching variants, it is important to remember that the rearrangements that occur will remove and destroy the intervening sequences, so only those heavy-chain constant regions that are found further downstream can be selected for. The order of the heavy-chain constant regions is μ, δ, γ_3, γ_1, γ_{2b}, γ_{2a}, ϵ, and α. Workers should also be certain they need these variants, as the assays are tedious. It may often be more advantageous to set up another fusion rather than isolate switch variants.

The most useful approach for most laboratories has been developed by Scharf and his colleagues (for a summary, see Spira et al. 1985). First, a suitable assay must be developed. Because of the large number of assays that must be performed, enzyme-linked assays are generally more useful. The assay for antibody capture on p. 180 can be easily adopted by changing the detection reagent to an IgG- or IgG_{2a}-specific rabbit anti-mouse immunoglobulin antibody. (Not all companies supply reagents that are sufficiently specific for these tests; one useful source is Southern Biotechnical Associates. All sources should be tested carefully before use.)

Hybridoma cells should be washed by centrifugation. Resuspend the cell pellet in medium supplemented with 20% fetal bovine serum at a density of 10^4 cells/ml and dispense 100 μl into the wells of 10 96-well microtiter plates. This yields approximately 1000 cells per well with about 1000 wells. Therefore, about 10^6 cells are being screened per assay. After the cells have grown, remove a sample of the tissue culture supernatant and screen for the presence of the IgG or IgG_{2a} antibodies. Between one and five positive wells may be seen. Choose the strongest positive, and transfer these cells to fresh medium. Continue passaging the cells until they are numerous enough to clone again. In the second round, the cells should be plated at 100 cells per well. The procedure is repeated and then the cells are plated at 10 cells per well. In the last round the cells are single-cell cloned using one of the techniques described on p. 219.

INTERSPECIES HYBRIDOMAS

Antibody-secreting cells isolated from one species but fused with myelomas from another species yield interspecies hybridomas. These types of fusions were common in the early years of hybridoma production. Often these hybrids would be formed by immunizing rats and fusing with mouse myeloma cells. This was done before good rat myeloma fusion partners were available. These fusions yield hybridomas that secrete rat antibodies, but the hybridoma cells cannot be grown conveniently as ascites tumors. Therefore, antibody production is almost entirely limited to tissue culture sources.

Although some important monoclonal antibodies have been produced using interspecies fusions, there seems little need for using these types of fusions today.

HUMAN HYBRIDOMAS

One of the most exciting areas for hybridoma research over the last 5 years has been the development of systems for the production of human hybridomas. Human monoclonal antibodies will be used extensively for clinical applications. Although this field has been marked by exciting publications announcing new breakthroughs, the actual progress in setting up the routine production of human hybridomas for laboratory use has been slow. For most research applications, producing human hybridomas still does not offer many, if any, advantages. The two most successful strategies that are used are standard fusions with human myeloma cells and the use of the Epstein-Barr virus (EBV) to transform antibody-secreting cells. One of the major problems in producing human hybridomas has been the lack of a suitable myeloma partner. Several of these lines have been isolated and are now in use.

The use of EBV-transformation to allow antibody-secreting cells to grow in standard tissue culture systems has solved some of the problems in human monoclonal antibody production. One unfortunate drawback of this approach is that the resultant transformants seldom secrete large amounts of antibodies. This has been overcome in some cases by fusing the EBV-transformed cell with a mouse myeloma cell line to allow the secretion of large amounts of antibodies. The combined use of EBV and secondary fusions points out two important aspects in hybridoma research. One is the use of other vectors to deliver important genetic information such as oncogenes. Second, if a particular hybrid does not possess all of the properties that are needed for a particular use, the line may be refused with other hybrids to achieve these properties.

There are several publications that describe progress in the isolation of human antibody-secreting cells, and these types of references should be checked for the details of producing human hybridomas.

FUTURE TRENDS

Few changes in the techniques used to produce hybridomas have been adopted since the original methods of Köhler and Milstein were reported. However, hybridoma construction is likely to change radically during the next 10 years. In several areas, preliminary work has already been reported that will form the basis for more widespread use of new techniques.

1. **In vitro immunizations** Although the first in vitro immunization procedures were described in the early 1980s, they have not come into common use. The two major advantages of in vitro immunizations are the small amount of antigen that is required (as low as 1 ng) and the lack of cellular regulation on the developing immune response. Both of these factors make in vitro immunizations a potentially powerful technology. They have not been widely used to date, because so far they do not allow the development of high-affinity antibodies and because many of the antibodies that are produced are from the IgM class.
2. **Electrofusion** PEG fusions routinely produce one viable hybridoma from 10^5 starting cells, and this may be below the needed efficiency. One method that is gaining more widespread use is fusing cells by applying high-voltage electrical gradients across cell populations—short bursts fuse adjacent membranes and yield hybrid cells. This method has been applied successfully to hybridoma production, and the higher fusion efficiency allows production of more hybrid cells. In general, this has not been important for most fusions, because hybridoma production is normally limited by the screening method rather than by the frequency of hybridoma production. As more rapid screening procedures are developed, this fusion method will become more important. Also, as techniques are developed that allow the selection of the desired antibody-secreting cell prior to fusion, this and other high-efficiency methods will become increasingly valuable.
3. **Retroviral vectors** Recombinant retroviral vectors hold the most promise for the efficient transformation of antibody-secreting cells. These vector systems can be engineered to deliver oncogenes into cells. However, the exact gene or combination of genes that will immortalize plasma cells but will not affect antibody secretion has not been determined. Also, because there will be little discrimination between the desired parental cells and undesired ones, this technology will be useful only when other methods of physically isolating the correct antibody-secreting cell are routinely used.

4. **Antigen-directed fusions** A number of methods are being developed that, prior to fusion, physically couple myeloma cells with cells that are secreting the desired antibodies. These techniques take advantage of the antigen-combining site of surface antibodies found on some secreting cells. This combining site is used as a target for a modified antigen that will also bind to myeloma cells. After fusion, the frequency of appearance of hybridomas secreting the desired antibodies is much higher than in undirected fusions.
5. **Fusion partners** More sophisticated methods of identifying cells that secrete the desired antibodies are being developed. Most of these methods use a fluorescence-activated cell sorter to identify and purify cells with surface immunoglobulins having the correct specificity. These cells then can either be fused with myeloma cells or transformed by other methods. These technologies will continue to improve, giving better and more refined choices for antibody selection prior to fusion or transformation. In addition, new myeloma fusion partners are constantly being described that have better properties for successful fusions.
6. **Defined medium** Many of the growth factors that are necessary for the cultivation of hybridomas have been identified, and several defined medium have been developed. These culture conditions allow hybridomas to be grown in medium that do not contain other immunoglobulins (often bovine), and the low levels of proteins in these solutions make purification of antibodies from the tissue culture supernatants easier.

PREPARING SPLENOCYTE FEEDER CELL CULTURES

Although splenocyte feeders can be used immediately, they are most effective when they are prepared approximately 1 day before the single-cell cloning. Because spleen cells do not grow in normal tissue culture conditions, they are lost during the subsequent expansion of the hybridoma cells. Use a female mouse of the same genetic background as your hybridoma.

1. Sacrifice the mouse. See your local authorities on animal handling for advice on the proper humane procedures. Remove the spleen aseptically from the mouse and place in a 100-mm tissue culture dish containing 5 ml of medium without serum (see p. 209). Trim off and discard any contaminating tissue from the spleen.

2. Tease apart the spleen using 19-gauge needles on 1.0-ml syringes. Continue to tease until most of the cells have been released and the spleen has been torn into very fine parts. Disrupt any cell clumps by pipetting. Transfer the cells and medium into a conical centrifuge tube leaving behind all of the larger pieces of tissue. Wash these clumps and the plate with an additional 5 ml of medium without serum and combine with the first 5 ml.

3. Allow the cell suspension to sit at room temperature for approximately 2 min. This will allow the larger cell clumps to settle to the bottom of the tube. Carefully remove the medium and cells avoiding the sediment, and transfer to 100 ml of medium with 10% fetal bovine serum prescreened to support hybridoma growth (1 spleen per 100 ml is about 10^8 cells/100 ml or 10^6/ml). Either use directly or prepare conditioned medium.

4. **Either:** To use directly for 96-well cloning (pp. 222–224), plate 50 μl of the spleen cell solution into each of the wells of a 96-well tissue culture dish. Allow to grow for 24 hr at 37°C.

   Or: To use directly for soft-agar cloning (p. 226), the medium with the feeder cells is used to dilute the hybridoma cell suspension prior to mixing with the soft-agar.

   Or: To prepare conditioned medium, transfer the splenocyte cell suspension to several tissue culture dishes. Place at 37°C in a CO_2 incubator for 3 days. Collect the cell suspension and remove the cells by centrifugation at 400*g* for 10 min. Filter sterilize and dispense in convenient sizes. Freeze at −70°C. Use the conditioned medium mixed 1:1 with medium supplemented with 20% FBS and 2× OPI.

NOTE

i. To avoid any possible problems with a particular spleen feeder culture, it may be best to combine several batches.

PREPARING FIBROBLAST FEEDER CELL CULTURES

Certain fibroblast cultures secrete the necessary factors to allow the growth of hybridoma cells at low plating densities. Early studies used fibroblast cultures that had been treated with mitomycin C or lethal doses of irradiation. Both of these treatments made it impossible for the feeder cells to contaminate future cultures of the hybridomas. More recently, this has been shown not to be necessary for fibroblast cultures that adhere strongly to the plastic tissue culture surface. Other studies have compared the ability of different fibroblast cells to support single-cell cultures of hyridoma cells and have found that the human diploid cells MRC 5 are the most effective in this test. These cells are not an established cell line, and so will need to be replaced in the future by another source. The MRC 5 cells are currently available from several sources including the American Type Culture Collection.

1. The MRC 5 cells are grown and maintained in Ham's F12 medium supplemented with 10% fetal bovine serum. They should be used at passages below 40.

2. Trypsinize the cells and count. Prepare a solution of 2×10^5 cells/ml of medium with 10% fetal bovine serum prescreened to support hybridoma growth.

3. For cloning using 96-well tissue culture dishes (pp. 222–224), add 50 μl of the cell suspension to the wells. Allow the cultures to grow for 1 day at 37°C.

   Or: For soft-agar cloning using 60- or 100-mm tissue culture dishes (p. 226), add 10 ml of the cell suspension to a 100-mm dish or 3 ml to a 60-mm dish. Allow the cells to adhere to the plastic overnight at 37°C. Remove the medium and add the soft agar hybridoma cell suspension to the plate.

SINGLE-CELL CLONING BY LIMITING DILUTION

Cloning hybridoma cells by limiting dilution is the easiest of the single-cell cloning techniques. Two approaches are given below, one rapid technique for generating cultures that are close to being single-cell cloned and one for single-cell cloning directly.

Even though every attempt is made to ensure that the cells are in a single-cell suspension prior to plating, there is no way to guarantee that the colonies do not arise from two cells that were stuck together. Therefore, limiting dilution cloning should be done at least twice to generate a clonal population.

Limiting Dilution (Rapid)

1. Using a multiwell pipettor (8-, 12-, or 96-well), add 50 μl of medium with 20% FBS and 2× OPI to each well of a 96-well plate. The wells should already contain 50 μl of feeder cells (pp. 220 or 221) giving 100 μl total volume/well.

2. The hybridomas should be growing rapidly. Remove 100 μl of the hybridoma cell suspension using a pipetman and transfer to the top left-hand well. Mix by pipetting.

3. Do 1 in 2 doubling dilutions down the left-hand row of the plate (8 wells, 7 dilution steps). Discard tip.

4. Do 1 in 2 doubling dilutions across the plate using an 8-well multi-pipetter.

5. Clones should be visible by microscopy after a few days and normally will be ready to screen after 7–10 days. Score the wells by microscopy. There should be a line running on a 45° diagonal that contains approximately the same number of clones per well. If the cells are nearly cloned when you start, screen only wells with one or two clones. If not, screen a selection of wells with multiple clones as well as all those with only one clone.

6. Select the best wells and either grow up or repeat the cloning procedure directly.

Limiting Dilution (Slow)

1. The hybridomas should be healthy and rapidly growing at the time of cloning. Prepare four dilution tubes with medium supplemented with 20% fetal bovine serum and 2× OPI for each cell to be cloned. Three tubes should have 2.7 ml and the fourth should have 3.0 ml.

2. Add 10 μl of the hybridoma cells to the tube containing the 3.0 ml of medium. Do 1 in 10 dilutions of the hybridomas by removing 0.3 ml and transferring into the 2.7-ml tubes.

3. Add 100 μl of each dilution into 24 of the wells of a 96-well tissue culture plate (24 wells/dilution; 4 dilutions/plate, i.e., one hybridoma/plate). The wells should already contain 50 μl of feeder cells (pp. 220 or 221), giving 150 μl total volume/well. If the cells from the highest dilution are plated first, then the pipet does not need to be changed during the plating.

   If many hybridomas are being cloned at the same time, it may be worthwhile to plate the dilutions by using a 10-ml or larger pipet. One drop from these pipets will deliver approximately 100 μl.

4. Clones will begin to appear in 4 days and should be ready to screen starting about days 7–10.

   Screens can be done from wells containing multiple clones as well as from wells containing only single clones.

*SINGLE-CELL CLONING BY PICKS**

Cloning hybridomas by picking a single cell from a growing culture is the only cloning method that ensures that clones arise from a single cell. During the cloning procedure, the cell is followed under the microscope to be certain that the clone comes from only one cell.

1. Add approximately 100 μl of medium with 20% FBS and 2× OPI to the wells of a 96-well plate (approximately 20 wells/hybrid). The wells should already contain 50 μl of feeder cells (pp. 220 or 221) giving 150 μl total volume/well.

2. At the time of the cloning all cells should be growing rapidly. Do serial 1 in 5 dilutions of the hybridoma cells in 60-mm dishes. Use about 0.3 ml into 1.2 ml; this will allow enough volume to cover the bottom of the plate, but not so deep as to make the pipetting difficult. Observe the cells under the microscope and choose a plate with well-separated cells.

3. Use a drawn out 50-μl capillary pipet connected to a mouth pipetting device with a 0.2-μm filter fitted in the line. Partially fill the pipet with complete medium from a separate plate without cells. While watching under the microscope, draw a single cell into the pipet. Move to an area of the plate without any cells and blow out the cell to make sure you have only one cell. Draw it up again and transfer to one of the wells with feeders. With practice, single-cell picks take about 1 min.

4. The clones should be ready to screen in 7–10 days.

*J. Wyke (pers. comm.).

NOTE

i. Because this technique demands working under the microscope on the open bench, one might expect contamination to be common. However, the only portion of the tissue culture medium that is exposed to the open air for long is the dish that you are picking from and you only transfer a very small volume at one time. So the chances of contamination are low. Needless to say, this technique should only be done in an area without drafts.

SINGLE-CELL CLONING BY GROWTH IN SOFT AGAR

Cloning of hybridoma cells in semisolid medium is one of the most commonly used methods for producing single-cell clones. The technique is easy, but, because it is performed in two stages, it does take longer than other methods. Not all cells will grow in soft agar, and there may be a bias on the type of colony that appears. However, most of the commonly used myeloma fusion partners have relatively good cloning efficiencies in soft agar, and consequently, so do most hybridomas.

Even though every attempt is made to ensure that the cells are in a single-cell suspension prior to plating, there is no way to guarantee that the colonies do not arise from two cells that were stuck together. Therefore, single-cell cloning in soft agar should be repeated at least twice before the cells are considered clonal.

1. Prior to cloning prepare 3% agarose (Seaprep 15/45, FML Corporation or equivalent) in H_2O suitable for tissue culture. Sterilize by autoclaving. This is stable for 6 months to 1 year.

   Prepare double-strength medium, normally from powdered medium. Add 100 μg/ml of gentamicin, and sterilize by filtration. Store at 4°C. Stable for about 1 month at 4°C, but at that time if fresh glutamine is added to 2 mM the shelf life can be extended to 3 months.

2. Melt agarose in a boiling water bath or in a microwave oven and cool to 37°C.

3. To the 2× medium add fetal bovine serum to 20% and OPI to 2×. Warm to 37°C in a water bath.

4. Cells should be healthy and growing rapidly at the time of cloning. The cells should be as free of clumps as possible. Do 1 in 10 dilutions of hybridomas in 1× medium. If not using feeders, the 1× medium is prepared by diluting a sample of the complete 2× medium with sterile H_2O. If using feeders that grow in suspension, the medium used for these dilutions should be the cell suspension from the feeder cell preparation (p. 220). If using fibroblast feeders (p. 221), these cells should be plated on the tissue culture dishes to be used for the cloning 24 hr earlier, and the 1× medium should be prepared by diluting the 2× complete medium.

5. Add 150 μl of cells from the dilutions between 10^5 and 10^2 cells/ml to 60-mm tissue culture plates (2 plates/dilution). Do not bother to count cells. If you are uncertain about the exact concentration of cells, it is easier to do an extra dilution than to count the cells.

6. Mix the 3% agarose and the 2× medium 1 : 1. Add 4 ml to each plate, and mix by pipetting.

7. Place the plates at 4°C for 45 min and then transfer to 37°C in a CO_2 incubator.

8. Macroscopic clones will appear beginning about day 10. Pick clones from the highest dilution that shows growth. Remove a plug of agarose containing the colony with a sterile Pasteur pipet. Transfer the plug to 1 ml of medium in a 24-well plate. Disperse the clone by pipetting.

9. Supernatants from these wells will normally be ready for screening 48–72 hr later.

NOTE

i. As an alternative, the cells may be grown in the dilution tubes themselves (Civin and Banquerigo 1983). Add 2 ml of the 1.5% agarose/medium solution to each tube and grow as described above.

Unstable Lines

If hybridomas continue to produce less than 100% positive wells, even after four or more single-cell cloning steps, the lines probably have an unstable assortment of chromosomes. If the antibodies produced by these cells are particularly valuable, extra work to save these lines may be necessary. Two strategies are used. In the first and most straightforward, the single-cell cloning is continued on a regular basis, trying to isolate a stable subclone. Perhaps surprisingly, this often works. The screening assays should be adjusted to screen not only for the presence of the appropriate antibody, but also for the levels of antibody produced. Wells that contain a stable subclone of the original should produce higher levels of antibodies. If the stable variant is generated early in the proliferation within a well, the differences in antibody production between the well containing the variant and those that do not will be significant. At this stage many workers stop screening with an antigen-specific assay and only screen for the level of mouse antibody produced (see p. 560 for examples). After a stable line is generated, the specificity of the antibody should be reestablished.

A second strategy is to refuse the important line with a myeloma and allow the chromosomes to reassort from the beginning, hoping to isolate the stable variant from this source. To date, most re-fusions have been done by standard techniques and extensive screening. However, the introduction of a selectable drug selection marker into a suitable myeloma cell line should make selection against the parental myelomas easier. The hybridoma would carry a functional HPRT gene, while the myeloma would carry, for example, a neomycin gene. Selection for both genes should yield only successful secondary hybridomas.

Contamination

During the early stages of the fusion, contamination will mean the loss of the well or the fusion; however in later stages, important hybridomas can sometimes be saved.

CONTAMINATION IN THE FUSION WELLS— A FEW WELLS ONLY

1. Contaminated wells can be identified by their unusual pH or turbidity. Confirm the presence of the contaminating organisms by observing under the microscope. Mark the wells.

2. Move to the tissue culture hood and carefully remove the lid. If the underside of the lid is damp, replace with a new lid. Dry the top and edges of the plate itself by aspiration before replacing. If there is contaminated medium on the lid, autoclave the whole plate without any further work.

3. Remove the medium from the contaminated well by aspiration. Try to avoid generating any aerosols. Add enough 10% bleach to the well to bring the level right to the rim. Allow it to sit for 2 min at room temperature.

4. Remove the bleach from the contaminated well by aspiration. Add enough ethanol to the well to bring the level right to the rim. Remove by aspiration and repeat.

5. Dry the well by aspiration.

CONTAMINATION IN THE FUSION WELLS—GROSS

1. Autoclave the plates.

CONTAMINATION OF A CLONED LINE

1. If the line has been frozen, it is easiest to go back to the most recent freeze down and thaw a fresh vial of the cells.

2. If the line has not been frozen, inject the cells into mice that have been primed for ascites production (p. 274). The animals must be of a compatible genetic background to your hybrids (e.g., BALB/c × BALB/c into BALB/c or BALB/c × C57Bl/B6 into BALB/c × C57B1/B6 F_1). If no mice have been primed with 0.5 ml of pristane the required 1 week in advance, inject 0.5 ml of Freund's adjuvant into the peritoneum. Wait 4 hr to 1 day and inject the hybridomas. Inject at least two mice for each contaminated culture.

3. When and if ascites develop, tap the fluid and transfer into a sterile centrifuge tube (see p. 274 for more information on ascites production).

4. Spin the ascites at 400g for 5 min at room temperature.

5. Remove the supernatant. Resuspend the cell pellet in 10 ml of medium supplemented with 10% fetal bovine serum and transfer to a tissue culture plate. The supernatant can be checked for production of the appropriate antibody. If positive, save for use.

6. Handle as for normal hybridomas, except keep the cells separate from the other cultures until there is little chance of the contamination reappearing.

The success rate may be as high as 80%.

NOTE

i. Animals injected with infected cultures should be kept isolated from the main animal colony.

Classing and Subclassing of Monoclonal Antibodies

Many techniques for using monoclonal antibodies require antibodies with specific properties. One set of these properties is unique to the individual antibody itself and includes such variables as specificity and affinity for the antigen. These properties all depend on differences in the antigen-combining domain of the antibody and can be assayed by comparing the properties of the monoclonal antibodies in tests that measure antigen binding activity.

A second set of important properties for monoclonal antibodies is determined by the structure of the remainder of the antibody, sequences encoded by the antibody common regions. These properties include the class or subclass of the heavy chain or the light chain. The different classes or subclasses will determine the affinity for important secondary reagents such as protein A (see p. 616). The type of heavy and light chain can be distinguished by simple immunochemical assays that measure the presence of the individual light- and heavy-chain polypeptides. This is normally achieved by raising antibodies specific for the different mouse heavy- and light-chain polypeptides (p. 622). The production of these antibodies is possible because the light- and heavy-chain polypeptides from different species are sufficiently different to allow them to be recognized as foreign antigens. Most often these anti-mouse immunoglobulin antibodies are raised in rabbits as polyclonal sera, and then the antibodies specific for a particular heavy or light chain are purified on immunoaffinity and immunodepletion columns. Although these chain-specific rabbit anti-mouse immunoglobulin antibodies can be made in the laboratory, it is normally easier to purchase them from commercial sources. There are a large number of different assays used, and some of the more common are listed below.

*DETERMINING THE CLASS AND SUBCLASS OF A MONOCLONAL ANTIBODY BY OUCHTERLONY DOUBLE-DIFFUSION ASSAYS**

Originally, the Ouchterlony double-diffusion assays were the most common method for determining class and subclass of a monoclonal antibody. They have been largely superseded by other techniques, but they still are useful, particularly when only a few assays will be performed. In these assays, samples of tissue culture supernatants (often concentrated tenfold) are pipetted into a well in a bed of agar. Class- and subclass-specific antisera are placed in other wells at equal distance from the test antibody. The two groups of antibodies diffuse into the agar. As they meet, immune complexes form, yielding increasing larger complexes as more antibodies combine. When large multimeric complexes form, the immune complexes will precipitate, forming a line of proteins that is either visible to the naked eye or that can be stained to increase the sensitivity. The precipitated proteins form what is referred to as a precipitin line.

1. Prepare a 10-ml sample of tissue culture supernatant from a hybridoma. Grow the cells in medium supplemented with 10% FBS and allow the culture to overgrow and die.

2. Spin the tissue culture supernatant at 1000*g* for 10 min. Collect the supernatant. If the supernatant is not clear of all debris, either filter it through a 0.45-μm filter (sterility is not important) or spin at 7000*g* for 15 min.

3. Concentrate the supernatant 10-fold using an ultrafiltration manifold. This is most easily done with adaptors that are designed to concentrate in the centrifuge. Many of the ultrafiltration specialty companies now supply these devices; follow the manufacturers' instructions. Remove the tissue culture supernatant when the 10 ml sample has been reduced to 1 ml.

   Tissue culture supernatants may also be concentrated by ammonium sulfate precipitation (p. 298).

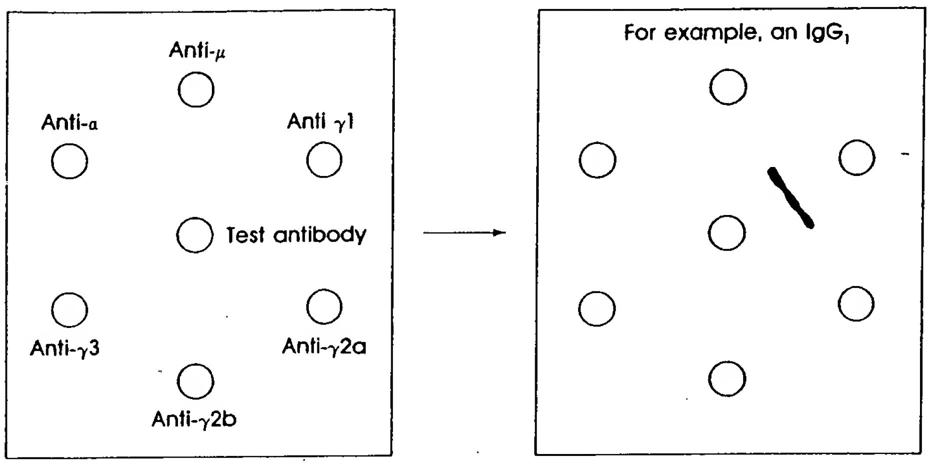
4. Prepare a 1.4% agarose solution in PBS with 5 mM EDTA. Melt the agarose in a boiling water bath or in a microwave. Cool to 45°C.

   Ouchterlony plates can also be purchased commercially.

5. On a level surface pipet 3 ml of the agarose solution onto the top of a 3 × 5-cm clean glass slide. The agarose should form a layer about 2 mM deep. The surface tension of the agarose should hold the agarose on the slide. Allow to harden at room temperature.

*Ouchterlony (1949).

6. Using a 200-μl capillary pipet or a commercial apparatus, carefully core vertical small holes in the agarose in a pattern that looks like this:



If the capillary pipet is attached to a pipetting device, a light suction while preparing the wells will allow the plugs to be withdrawn easily.

7. Add 5 μl of rabbit anti-mouse immunoglobulin sera specific for the various classes, subclasses, or light chains to each of the wells in the outer ring.

8. Add 5 μl of the concentrated tissue culture supernatant to the middle well.

9. Incubate in a humid atmosphere overnight at room temperature.

10. Score positive reactions by the appearance of a precipitin line between the wells with reactive antibodies.

NOTE

i. The sensitivity of these assays can be increased by staining the bands with Coomassie brilliant blue. Cover the gel with wet filter paper and place in a 50°C oven. Incubate until dry. Wet the paper and remove from the gel. Wash for 30 min in several changes of PBS. Repeat the drying procedure. Stain with Coomassie for 15 min (p. 649). Destain in 7% acetic acid, 25% methanol.

DETERMINING THE CLASS AND SUBCLASS OF MONOCLONAL ANTIBODIES USING ANTIBODY CAPTURE ON ANTIGEN-COATED PLATES

Any of the assays used to screen hybridoma fusions that detect antibodies with a secondary anti-mouse immunoglobulin antibody can be adapted to screen for class or subclass. For example, if the detection method used ^{125}I-labeled rabbit anti-mouse immunoglobulin to locate antibodies bound to the antigen, then substituting anti-class or subclass-specific antibodies for the ^{125}I-reagent will identify the type of heavy chains. An example of these types of reactions is given below using an antigen bound to 96-well PVC plates, but similar tests could be developed for any of the antibody capture assays.

1. Prepare a solution of approximately 2 μg/ml of the antigen in 10 mM sodium phosphate (pH 7.0). Higher concentrations will speed the binding of antigen to the PVC, but the capacity of the plastic is only about 300 ng/cm^2, so the extra protein will not bind to the PVC. However, if higher concentrations are used, the protein solution can be saved and used again. Many workers prefer to use 50 mM carbonate buffer (pH 9.0) in place of the phosphate buffer.

2. Add 50 μl of antigen to each well. Incubate at room temperature for 2 hr or overnight at 4°C.

3. Remove the contents of the well. If the solution will not be saved, it can be removed by aspiration or by flicking the liquid into a suitable waste container.

4. Wash the plate twice with PBS. A 500-ml squirt bottle is convenient.

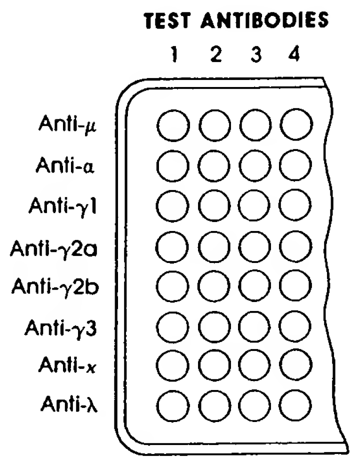
5. Fill the wells with 3% BSA/PBS (no sodium azide). Incubate for at least 2 hr at room temperature.

6. Wash the plate twice with PBS.

7. Add 50 μl of each tissue culture supernatant to be tested to every well of a vertical row (8 wells/test). Incubate 1 hr at room temperature.

8. Wash the plate twice with PBS.

9. Add 50 μl of 3% BSA/PBS (without sodium azide) containing a dilution of horseradish peroxidase-labeled rabbit anti-mouse immunoglobulin class- or subclass-specific antibody to each well as shown below:



Incubate 1 hr at room temperature. (Horseradish peroxidase-labeled reagents can be purchased or prepared as described on p. 344. Most commercial reagents should be diluted 1 in 1000 to 1 in 5000. Try several dilutions in preliminary tests and choose the best.)

10. Wash the plate with PBS three times.

11. During the final washes prepare the TMB substrate solution. Dissolve 0.1 mg of 3′,3′,5′,5′-tetramethylbenzidine (TMB) in 0.1 ml of dimethylsulfoxide. Add 9.9 ml of 0.1 M sodium acetate (pH 6.0). Filter through Whatman No. 1 or equivalent. Add hydrogen peroxide to a final concentration of 0.01%. This is sufficient for two 96-well plates. (Hydrogen peroxide is normally supplied as a 30% solution. After opening, it should be stored at 4°C, where it is stable for 1 month.)

12. After the final wash in PBS, add 50 μl of the substrate solution to each microtiter well.

13. Incubate for 10–30 min at room temperature. Positives appear pale blue.

14. Add 50 μl of stop solution, 1 M H_2SO_4, to every well. Positives now appear bright yellow. To quantitate the binding, read the results at 450 nm.

NOTE

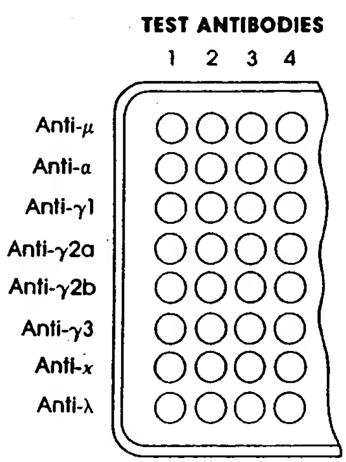
i. Do not include sodium azide in solutions when horseradish peroxidase is used for detection.

DETERMINING THE CLASS AND SUBCLASS OF MONOCLONAL ANTIBODIES USING ANTIBODY CAPTURE ON ANTI-Ig ANTIBODIES

One of the easiest methods for determining the class and subclass of a monoclonal antibody is to bind class- or subclass-specific antibodies to the wells of a polyvinylchloride (PVC) plate. The test monoclonal antibody is added to each well, but will bind only to wells coated with antibodies that are specific for its subclass or class. These bound antibodies are detected using a secondary antibody specific for all mouse antibodies.

1. Purify the antibodies from rabbit anti-mouse immunoglobulin class- or subclass-specific antibodies. Techniques for these purifications are discussed in Chapter 8. For most purposes, protein A beads are probably the easiest to use. (Rabbit anti-mouse immunoglobulin class- and subclass-specific sera can be purchased from several suppliers.)

2. After purification dilute the antibodies to 20 μg/ml in PBS. Add 50 μl to the wells of a PVC plate in the pattern below. Each monoclonal antibody being tested will need one row.



3. Incubate for 2 hr to overnight at room temperature in a humid atmostphere.

4. Remove the antibodies and save for future use. The antibodies can be reused approximately five times.

5. Fill the wells with 3% BSA/PBS with 0.02% sodium azide. Incubate for 2 hr or overnight at room temperature.

6. Wash three times with PBS. Add 50 μl of tissue culture supernatant from each hybridoma to the appropriate wells.

7. Incubate at room temperature for 2 hr in a humid atmosphere. Shake out the unbound antibody, and wash three times with PBS.

8. Add 50,000 cpm of ^{125}I-labeled rabbit anti-mouse immunoglobulin antibody to each well (diluted in 3% BSA/PBS with 0.02% sodium azide).

9. Incubate for 2 hr at room temperature in a humid atmosphere. Discard the iodinated antibodies in an appropriate waste container.

10. Wash the wells three times with PBS. Cut the wells from the plate and count in a gamma-counter.

NOTE

i. Other detection methods can be substituted for the iodinated antibodies. Common alternatives include enzyme-labeled reagents.

Selecting Class-switch Variants

During the normal development of a humoral response, the predominant class of antibodies that are produced changes, beginning primarily with IgMs and developing into IgGs. These changes and others like them occur by genetic rearrangements that move the coding region for the antigen binding site from just upstream of the IgM-specific region to the IgG region. These events are described in detail in Chapter 2 (p. 7). These rearrangements help the host animal tailor the immune response to the various types of infection. The different classes and subclasses of antibodies also have properties that make them more or less useful in various immunochemical techniques. These differences make the preparation of antibodies of certain classes or subclasses very valuable.

Recently, it has been shown that a process that appears similar to the natural class and subclass switching occurs in vitro, although at a very low frequency. Therefore, any population of hybridomas will have a small proportion of cells secreting antibodies with a different class or subclass of antibody. The antigen binding site will be identical in these antibodies. If these cells can be identified and cloned, then antibodies with the same antigen binding site but with different class or subclass properties can be isolated. These "shift variants" generally are useful in one of two cases, either switching from IgM to IgG or from IgG_1 to IgG_{2a}. Often these switches are used to produce antibodies that bind with higher affinity to protein A.

When trying to identify any class or subclass switching variants, it is important to remember that the rearrangements that occur will remove and destroy the intervening sequences, so only those heavy-chain constant regions that are found further downstream can be selected for. The order of the heavy-chain constant regions is μ, δ, γ_3, γ_1, γ_{2b}, γ_{2a}, ϵ, and α. Workers should also be certain they need these variants, as the assays are tedious. It may often be more advantageous to set up another fusion rather than isolate switch variants.

The most useful approach for most laboratories has been developed by Scharf and his colleagues (for a summary, see Spira et al. 1985). First, a suitable assay must be developed. Because of the large number of assays that must be performed, enzyme-linked assays are generally more useful. The assay for antibody capture on p. 180 can be easily adopted by changing the detection reagent to an IgG- or IgG_{2a}-specific rabbit anti-mouse immunoglobulin antibody. (Not all companies supply reagents that are sufficiently specific for these tests; one useful source is Southern Biotechnical Associates. All sources should be tested carefully before use.)

INTERSPECIES HYBRIDOMAS

Antibody-secreting cells isolated from one species but fused with myelomas from another species yield interspecies hybridomas. These types of fusions were common in the early years of hybridoma production. Often these hybrids would be formed by immunizing rats and fusing with mouse myeloma cells. This was done before good rat myeloma fusion partners were available. These fusions yield hybridomas that secrete rat antibodies, but the hybridoma cells cannot be grown conveniently as ascites tumors. Therefore, antibody production is almost entirely limited to tissue culture sources.

Although some important monoclonal antibodies have been produced using interspecies fusions, there seems little need for using these types of fusions today.

HUMAN HYBRIDOMAS

One of the most exciting areas for hybridoma research over the last 5 years has been the development of systems for the production of human hybridomas. Human monoclonal antibodies will be used extensively for clinical applications. Although this field has been marked by exciting publications announcing new breakthroughs, the actual progress in setting up the routine production of human hybridomas for laboratory use has been slow. For most research applications, producing human hybridomas still does not offer many, if any, advantages. The two most successful strategies that are used are standard fusions with human myeloma cells and the use of the Epstein-Barr virus (EBV) to transform antibody-secreting cells. One of the major problems in producing human hybridomas has been the lack of a suitable myeloma partner. Several of these lines have been isolated and are now in use.

The use of EBV-transformation to allow antibody-secreting cells to grow in standard tissue culture systems has solved some of the problems in human monoclonal antibody production. One unfortunate drawback of this approach is that the resultant transformants seldom secrete large amounts of antibodies. This has been overcome in some cases by fusing the EBV-transformed cell with a mouse myeloma cell line to allow the secretion of large amounts of antibodies. The combined use of EBV and secondary fusions points out two important aspects in hybridoma research. One is the use of other vectors to deliver important genetic information such as oncogenes. Second, if a particular hybrid does not possess all of the properties that are needed for a particular use, the line may be refused with other hybrids to achieve these properties.

There are several publications that describe progress in the isolation of human antibody-secreting cells, and these types of references should be checked for the details of producing human hybridomas.

FUTURE TRENDS

Few changes in the techniques used to produce hybridomas have been adopted since the original methods of Köhler and Milstein were reported. However, hybridoma construction is likely to change radically during the next 10 years. In several areas, preliminary work has already been reported that will form the basis for more widespread use of new techniques.

1. **In vitro immunizations** Although the first in vitro immunization procedures were described in the early 1980s, they have not come into common use. The two major advantages of in vitro immunizations are the small amount of antigen that is required (as low as 1 ng) and the lack of cellular regulation on the developing immune response. Both of these factors make in vitro immunizations a potentially powerful technology. They have not been widely used to date, because so far they do not allow the development of high-affinity antibodies and because many of the antibodies that are produced are from the IgM class.
2. **Electrofusion** PEG fusions routinely produce one viable hybridoma from 10^5 starting cells, and this may be below the needed efficiency. One method that is gaining more widespread use is fusing cells by applying high-voltage electrical gradients across cell populations—short bursts fuse adjacent membranes and yield hybrid cells. This method has been applied successfully to hybridoma production, and the higher fusion efficiency allows production of more hybrid cells. In general, this has not been important for most fusions, because hybridoma production is normally limited by the screening method rather than by the frequency of hybridoma production. As more rapid screening procedures are developed, this fusion method will become more important. Also, as techniques are developed that allow the selection of the desired antibody-secreting cell prior to fusion, this and other high-efficiency methods will become increasingly valuable.
3. **Retroviral vectors** Recombinant retroviral vectors hold the most promise for the efficient transformation of antibody-secreting cells. These vector systems can be engineered to deliver oncogenes into cells. However, the exact gene or combination of genes that will immortalize plasma cells but will not affect antibody secretion has not been determined. Also, because there will be little discrimination between the desired parental cells and undesired ones, this technology will be useful only when other methods of physically isolating the correct antibody-secreting cell are routinely used.

4. **Antigen-directed fusions** A number of methods are being developed that, prior to fusion, physically couple myeloma cells with cells that are secreting the desired antibodies. These techniques take advantage of the antigen-combining site of surface antibodies found on some secreting cells. This combining site is used as a target for a modified antigen that will also bind to myeloma cells. After fusion, the frequency of appearance of hybridomas secreting the desired antibodies is much higher than in undirected fusions.
5. **Fusion partners** More sophisticated methods of identifying cells that secrete the desired antibodies are being developed. Most of these methods use a fluorescence-activated cell sorter to identify and purify cells with surface immunoglobulins having the correct specificity. These cells then can either be fused with myeloma cells or transformed by other methods. These technologies will continue to improve, giving better and more refined choices for antibody selection prior to fusion or transformation. In addition, new myeloma fusion partners are constantly being described that have better properties for successful fusions.
6. **Defined medium** Many of the growth factors that are necessary for the cultivation of hybridomas have been identified, and several defined medium have been developed. These culture conditions allow hybridomas to be grown in medium that do not contain other immunoglobulins (often bovine), and the low levels of proteins in these solutions make purification of antibodies from the tissue culture supernatants easier.

4.1-527

RECEIVED

OCT 04 2004

IAN C. McLEOD

The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

Paper No. 13

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte LINDA S. MANSFIELD, MARY G. ROSSANO, ALICE J. MURPHY and RUTH VRABLE

Appeal No. 2003-1919
Application No. 09/670,355

MAILED

SEP 30 2004

U.S. PATENT AND TRADEMARK OFFICE
BOARD OF PATENT APPEALS
AND INTERFERENCES

ON BRIEF

Before WILLIAM F. SMITH, GRIMES, and GREEN, Administrative Patent Judges.

GREEN, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 10-12, 18-20, 44, 45 and 47-52. Claims 10 and 51 are representative of the subject matter on appeal, and read as follows:

> 10. A vaccine for protecting an equid from a Sarcocystis neurona infection comprising a DNA from Sarcocystis neurona that encodes at least a 16 $\pm$ 4 kDa antigen and/or 30 $\pm$ 4 kDa antigen of Sarcocystis neurona.

Co: 9.

> 51. A vaccine composition which comprises an effective immunizing amount of DNA derived from Sarcocystis neurona capable of inducing an antibody immune response, and a pharmacologically acceptable carrier.

Claims 10-12, 18-20, 44, 45 and 47-52 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention, i.e., lack of adequate written description. In addition, claims 10-12, 18-20, 44-45 and 47-52 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to enable one skilled in the art to which it pertains, or with which it is most nearly connected, to make and/or use the invention, i.e., lack of enablement. Finally, claims 51 and 52 stand rejected under 35 U.S.C. § 112, second paragraph. After careful review of the record and consideration of the issues before us, we affirm the rejection of claims 10-12, 18-20, 44-45 and 47-52 under 35 U.S.C. § 112, first paragraph, for lack of adequate written description, and the rejection of claims 51 and 52 under 35 U.S.C. § 112, second paragraph, and decline to reach the merits of the rejection of claims 10-12, 18-20, 44-45 and 47-52 under 35 U.S.C. § 112, first paragraph, for lack enablement.

DISCUSSION

1. Rejection under 35 U.S.C. § 112, first paragraph, written description

Claims 10-12, 1-20, 44, 45 and 47-52 stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter that was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventor(s), at the time the application was filed, had possession of the claimed invention, i.e., lack of adequate written description.

According to the rejection, "[r]eview of the present specification, the art of record, and a search of the sequence databases for polynucleotides and/or polypeptide sequences of 16(±4) kD antigen and the 30(±4) kD antigen indicate that these sequences have not been identified nor described." Examiner's Answer, page 4. The rejection further contends "the limitation 'at least' in the claims does not limit the invention to 16(±4) kD and/or 30(±4) kD antigen of S. neurona and broadly reads on any antigen that is not disclosed. The specification describes general methods of cloning cDNA sequences from expression libraries; however, the sequences obtained by this method for 16(±4) kD and/or 30(±4) kD antigen are not disclosed." Id. at 4-5. The rejection concludes that "the claimed invention as a whole is not adequately described and is not conventional in the art as of Appellants' effective filing date." Id. at 5 (emphasis in original).

With respect to the issue of conception in the context of an interference count, the Court of Appeals for the Federal Circuit, our reviewing court, has stated that "irrespective of the complexity or simplicity of the method of isolation employed, conception of a DNA, like conception of any chemical substance requires a definition of that substance other than by its functional utility." Fiers v. Revel, 984 F.2d 1164, 1169, 25 USPQ2d 1601, 1604 (Fed. Cir. 1993). The court specifically rejected Fiers' argument "that the existence of a workable method for preparing a DNA establishes conception of that material." Id.

In Enzo Biochem, Inc. v. Gen-Probe Inc., 296 F.3d 1316, 63 USPQ2d 1602 (Fed. Cir. 2002), in determining whether or not a claim to a nucleotide sequence met the written description requirement, the court adopted a portion of the Guidelines proffered by the United States Patent and Trademark Office (USPTO). The court stated that:

> The written description requirement can be met by "showing that an invention is complete by disclosure of sufficiently detailed, relevant identifying characteristics . . . i.e., complete or partial structure, other physical and/or chemical properties, functional characteristics when coupled with a known or disclosed correlation between function and structure, or some combination of characteristics.

Enzo Biochem, 296 F.3d at 1324, 63 USPQ2d at 1613 (citations omitted).

In construing the above requirement, the court in In re Wallach, 378 F.3d 1330, 71 USPQ2d 1939 (Fed. Cir. 2004), recognized "that the written description requirement can in some cases be satisfied by functional description." Id., 378

F.3d at 1335. The court held, however, that

> such functional description can be sufficient only if there is also a structure-function relationship known to those of ordinary skill in the art. As we explained above, such a well-known relationship exists between a nucleic acid molecule's structure and its function in encoding a particular amino acid sequence: Given the amino acid sequence, one can determine the chemical structure of all nucleic acid molecules that can serve the function of encoding that sequence. Without that sequence, however, or with only a partial sequence, those structures cannot be determined and the written description requirement is consequently not met.

Id.

In the instant case, as noted by the rejection, neither the disclosure as filed, nor the prior art, discloses any sequence, either amino acid or nucleic, for either the 16(±4) kD and/or 30(±4) kD antigens. Consequently, the written description requirement is not met, and the rejection is affirmed.

Appellants argue with respect to the rejection of claims 10-12, 18-20, 44, 45 and 47-50 that they had "possession of Sarcocystis neurona which contains DNA encoding the 16 ±4 and 30 ±4 antigens. Thus, the applicants have possession of Sarcocystis neurona DNA encoding the 16 ±4 and 30 ±4 antigens." Appeal Brief, page 7. Appellants argue further that "[c]onstructing and screening an expression library for clones containing DNA encoding a particular protein is routine in the art," and thus "a person of ordinary skill in the art following the applicants' disclosure would have a high expectation of success of recovering clones from an expression library that express the 16 ±4 or 30 ±4

antigens using the antibodies against the 16 ±4 and 30 ±4 antigens prepared as taught in Example 1." Id. at 8.

Appellants' arguments are not convincing. First, the fact that appellants had possession of Sarcocystis neurona is not sufficient to provide possession of DNA that encodes the 16 ±4 and 30 ±4 antigens. As noted above, even a partial amino acid sequence of the 16 ±4 and 30 ±4 antigens, which would necessarily require possession of the source of the DNA, i.e., possession of Sarcocystis neurona, would not be sufficient to provide written description support for the claimed DNA encoding the 16 ±4 and 30 ±4 antigens. In addition, as also discussed above, the existence of a workable method to obtain the DNA sequence is also not sufficient to demonstrate written description support.

With respect to claims 51 and 52, appellants argue that appellants have possession of Sarcocystis neurona DNA, which "would be expected to encode a plurality of antigens, including the 16 ±4 and 30 ±4 antigens. Therefore, when the DNA is inoculated into a horse, the antigens encoded thereon are expressed in the horse." Appeal Brief, page 10. According to appellants, "[c]laims 51 and 52 do not depend on knowing the DNA sequences encoding the plurality of antigens. The claims merely require that the DNA encode one or more Sarcocystis neurona antigens. Thus, the DNA can be the entire Sarcocystis neurona genome(intact or fragmented) or particular DNA fragments therefrom." Id. at 11.

The above argument is also not found to be convincing. The disclosure as filed does not provide written description support for the use of the entire Sarcocystis neurona genome (intact or fragmented) or particular DNA fragments therefrom as a DNA vaccine. The written description is limited to a "DNA vaccine that contains or expresses at least one epitope of an antigen that has an amino acid sequence substantially similar to a unique 16 ($\pm$4kDa) antigen and/or 30 ($\pm$4) kDa antigen of Sarcocystis neurona." Specification, page 1 (Field of the Invention); see also pages 5, 17, 24 and 26. Thus, the rejection of claims 51 and 52 under 35 U.S.C. § 112, first paragraph, for lack of adequate written description, is affirmed for the reasons set forth supra with respect to the discussion of claims 10-12, 18-20, 44, 45 and 47-50.

2. Rejection under 35 U.S.C. § 112, second paragraph

Claims 51 and 52 stand rejected under 35 U.S.C. § 112, second paragraph, "as being vague and indefinite in the recitation of 'derived'. Is this DNA isolated from S. neurona?" Examiner's Answer, page 9.

This rejection is affirmed in view of appellants' statement that they will amend the term "derived" to "isolated." See Appeal Brief, page 20.

CONCLUSION

The rejection of claims 10-12, 1-20, 44, 45 and 47-52 under 35 U.S.C. § 112, first paragraph, for lack of adequate written description, and the rejection of claims 51 and 52 under 35 U.S.C. § 112, second paragraph are affirmed. Because we affirm the rejection under 35 U.S.C. § 112, first paragraph, on the

basis of lack of adequate written description, we decline to reach the merits of the rejection under 35 U.S.C. § 112, first paragraph, for lack enablement.

No time period for taking any subsequent action in connection with this appeal may be extended under 37 CFR § 1.136(a).

AFFIRMED

William F. Smith Administrative Patent Judge	)
Eric Grimes Administrative Patent Judge	) BOARD OF PATENT) APPEALS AND) INTERFERENCES
Lora M. Green Administrative Patent Judge	)

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4.1-526

The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Ex parte LINDA S. MANSFIELD, MARY G. ROSSANO, ALICE J. MURPHY and RUTH A. VRABLE

Appeal No. 2005-2386
Application No. 09/670,096

ON BRIEF

Before GRIMES, GREEN, and LEBOVITZ Administrative Patent Judges.

GREEN, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 1, 2 and 21,[1] all of the pending claims, which are reproduced below:

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[1] An amendment after final was filed concurrently with the Appeal Brief, dated August 20, 2004, and stamped August 23, 2004. Appellants state in the Appeal Brief that an Amendment was filed August 20, 2004, and in response, the examiner in the Examiner's Answer merely states that appellants' statement is correct, but does not explicitely state that the amendment was entered. But because the rejection of claim 2 under 35 U.S.C. § 112, second paragraph, for lack of antecedent basis was withdrawn, and the amendment after final remedied that issue, we infer that the amendment was entered. Thus, the claims as reproduced here are as amended by the August 23, 2004, amendment after final.

c: Mansfield
Hudson
6/6/06

1. A composition for treating an equid infected with Sarcocystis neurona comprising a mixture of isolated antibodies against a 16 ±4 kDa antigen of Sarcocystis neurona and isolated antibodies against a 30 ±4 kDa antigen of Sarcocystis neurona wherein the antibodies are from serum of an animal immunized with the antigen and wherein the mixture is in a pharmaceutically acceptable carrier.

2. The method of claim 21 wherein the antibodies are monoclonal antibodies.

21. A method for treating an equid infected with Sarcocystis neurona comprising:

(a) providing a mixture of antibodies against a 16 ±4 kDa antigen and a 30 ±4 kDa antigen, both of which are specific to Sarcocystis neurona, wherein the antibodies are selected from the group consisting of polyclonal antibodies from serum from an animal immunized with the antigen and monoclonal antibodies from a hybridoma, and wherein the antibodies are in a pharmaceutically acceptable carrier; and

(b) inoculating the equid with the antibodies in the carrier to treat the equid.

The claims stand rejected under 35 U.S.C. § 112, first paragraph, as containing subject matter that was not described in the specification in such a way so as to enable one skilled in the art to which it pertains or with which it is most nearly connected to make and/or use the invention. After careful review of the record and consideration of the issue before us, we reverse.

DISCUSSION

Claims 1, 2 and 21 stand rejected under 35 U.S.C. § 112, first paragraph, for lack of enablement.

"[A] specification disclosure which contains a teaching of the manner and process of making and using the invention in terms which correspond in scope to those used in describing and defining the subject matter sought to be patented must be taken as in compliance with the enabling requirement of the first

paragraph of § 112 unless there is reason to doubt the objective truth of the statements contained therein which must be relied on for enabling support." In re Marzocchi, 439 F.2d 220, 223, 169 USPQ 367, 369 (CCPA 1971) (emphasis in original). "[It] is incumbent upon the Patent Office, whenever a rejection on this basis is made, to explain why it doubts the truth or accuracy of any statement in a supporting disclosure and to back up assertions of its own with acceptable evidence or reasoning which is inconsistent with the contested statement." Id. at 224, 169 USPQ at 370. Here, the examiner has not provided "acceptable evidence or reasoning which is inconsistent" with the specification, and therefore has not met the initial burden of showing nonenablement.

In making the enablement rejection, the examiner engages in the analysis of the factors as set forth in In re Wands, 858 F.2d 731, 8 USPQ2d 1400 (Fed. Cir. 1988). Examiner's Answer, page 5.

The examiner notes that given the high rate of exposure of horses to S. neurona and the low incidence of clinical equine protozoal myeloencephalitis (EPM), "indicate[s] that most horses develop effective immunity (no clinical symptoms of disease) that may prevent merozoite entry into the central nervous system." Id. at 6. The examiner goes on to state that the pathogenesis of the disease is not fully understood, and that clinical manifestations of the disease only occur in a small percentage of seropositive horses, citing Cutler[2] in noting

[2] Cutler et al. (Cutler), "Immunoconversion against Sarcocystis neurona in normal and dexamethasone-treated horses challenged with S. neurona sporocysts," Veterinary Parasitology, Vol. 95, pp. 197-210 (2001).

that "it is important and necessary to identify factors that govern progression from an apparent infection to clinically evident neurological disease, EPM . . . in horses." Id.

According to the examiner, "[t]he treatment of S. neurona infection in an equid with antibodies is highly complex and unpredictable because relative to the infection, the development of clinical spreading of the disease i.e., merozoite entry into the central nervous system crossing blood brain barrier is not known as most of the horses develop immunity without EPM." Examiner's Answer, page 6. As "the prior art does not teach administration of a mixture of isolated antibodies against a 16 kD antigen of S. neurona and isolated antibodies against a 30 kD antigen of S. neurona to an infected horse with EPM which would resolve the infection in CNS[,] . . . [t]hus there is a lack of understanding in the art with respect to the pathogenesis of S. neurona infection in horses that develop EPM." Id. at 7.

The examiner also relies on Liang 1998[3] to support the proposition that "not all antibodies generated during infection will neutralize the merozites." Examiner's Answer, page 7. The examiner asserts that from Liang 1998 it appears that extended exposure to antiserum appears to be necessary, and that in vitro data do not necessarily correlate to the results that will be obtained in vivo. See id. Moreover, according to the examiner, "it is unclear whether such

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[3] Liang et al. (Liang 1998), "Evidence that Surface Proteins Sn14 and Sn16 of *Sarcocystis neurona* Merozoites Are Involved in Infection and Immunity," Infection and Immunity, Vol. 66, No. 5, pp. 1834-1838 (1998).

an immunotherapy can be used to treat all horses that are infected with S. neurona." Id. at 8. The examiner is also concerned that the specific antibodies used in the claimed immunotherapeutic methods are not characterized. See id.

We do not find that the examiner has provided evidence and/or reasoning that the claims are not enabled by the specification. As noted by appellants, "since many horses exposed to Sarcocystis neurona do not have clinical signs of EPM but have immunity to Sarcocystis neurona the serum antibodies are likely effective for protecting against the parasite." Appeal Brief, page 9. Given that, as further noted by appellants, "it would appear to be reasonable to believe that horses with EPM have an inadequate immune response to the parasite which is not sufficient to prevent entry of the parasite into the CNS and that boosting the immune response with antibodies against the 16 and 30 kDa antigens might provide a sufficient boost to an infected horse's immune response to inhibit entry of the parasite into the CSF." Id. at 16.

Moreover, Liang 1998 teaches that Sarcocystis neurona is sensitive to specific antibodies, and thus does not support the examiner's contention that the claims are not enabled. In regard to the examiner's statement that "it is unclear whether such an immunotherapy can be used to treat all horses that are infected with S. neurona," there is no requirement that the claimed method work with all horses that are infected with S. neurona.

In addition, Appellants submitted a declaration on April 1, 2003, Appendix B to the Appeal Brief, demonstrating that both the 16 and 30 kDa antigens

appeared to be more neutralizing than either antibody alone. See Appeal Brief, page 14. In response, the examiner argues that although "[t]he Declaration provides evidence that CSF from infected horses contains antibodies to 16kD and 30kD [antigens] and such antibodies neutralize the merozoites in vitro (neutralization assays) only," the declaration "does not provide any evidence that the claimed composition comprising said antibodies are useful for treating an equid infected with S. neurona." Examiner's Answer, page 13.

Thus, the examiner's principal concern appears to be that the specification provides no in vivo examples of treating a horse. See, e.g., Examiner's Answer, pages 6 and 8. The examiner notes that the specification "only discloses that multiple isolates of merozoites have been obtained by culturing sporozoites from opossum," id. at 8, and that "[t]he specification . . . provides no working examples demonstrating . . . enablement for the claimed composition or a method that is required in this under developed art. The specification only teaches culturing sporocysts and merozoites," id. at 9.

The presence or absence of a working example, however, is not determinative on the issue of enablement. It is just one factor that is to be weighed with the other factors. In the case at issue, the examiner has not met the burden of demonstrating that the specification does not enable the claims, and the rejection under 35 U.S.C. § 112, first paragraph, for lack of enablement, is reversed.

OTHER ISSUES

Appellants' and the examiner's attention is directed to related Appeal Number 2004-1976, U.S.S.N. 09/669,843. That appeal contained a claim to:

> A monoclonal mixture comprising an antibody that selectively binds to a 16 ± 4 kDa antigen of Sarcocystis neurona and a monoclonal antibody that selectively binds to a 30 ± 4 kDa antigen of Sarcocystis neurona wherein the antigens are separately isolated from Sarcocystis neurona merozoites by two-dimensional polyacrylamide gel electrophoresis and separately used to produce hybridomas which produce the monoclonal antibodies for the mixture.

In the 2004-1976 appeal, we affirmed a rejection under 35 U.S.C. § 103(a) over the combination of Liang 1998 or Liang 1997[4] and Harlow.[5]

Similarly, in the instant appeal, claim 1 is directed to:

A composition for treating an equid infected with Sarcocystis neurona comprising a mixture of isolated antibodies against a 16 ±4 kDa antigen of Sarcocystis neurona and isolated antibodies against a 30 ±4 kDa antigen of Sarcocystis neurona wherein the antibodies are from serum of an animal immunized with the antigen and wherein the mixture is in a pharmaceutically acceptable carrier.

The recitation of "for treating an equid infected with Sarcocystis neurona" is a statement of intended use, and not a patentable limitation. See In re

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[4] Liang et al. (Liang 1997), "Micropreparative High Resolution Purification of Proteins by a Combination of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, Isoelectric Focusing, and Membrane Blotting," Anal. Biochem., Vol. 250, pp. 61-65 (1997).

[5] Harlow et al. (Harlow, Antibodies, A laboratory Manual, Chapter 6, Col Spring Harbor Press (1988).

Schreiber, 128 F.3d 1473, 1477, 44 USPQ2d 1429, 1431 (Fed. Cir. 1997). Upon return of the appeal, the examiner may wish to consider the patentability of instant claim 1 in view of the references and the rejection as set forth in Appeal Number 2004-1976.

CONCLUSION

Because the examiner has not set forth a prima facie case of unpatentability, the rejection of claims 1, 2 and 21 under 35 U.S.C. § 112, first paragraph, for lack of enablement, is reversed. Upon receipt of the case, however, the examiner may wish to consider the patentability of claim 1 in view of the rejection under 35 U.S.C. § 103(a) as set forth in related Appeal 2004-1976.

REVERSED

Eric Grimes Administrative Patent Judge	
	BOARD OF PATENT
Lora M. Green Administrative Patent Judge	APPEALS AND
	INTERFERENCES
Richard M. Lebovitz Administrative Patent Judge	

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The opinion in support of the decision being entered today was not written for publication and is not binding precedent of the Board.

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE BOARD OF PATENT APPEALS
AND INTERFERENCES

Ex parte LINDA S. MANSFIELD, MARY G. ROSSANO,
ALICE J. MURPHY and RUTH A. VRABLE

Appeal No. 2004-1976
Application No. 09/669,843

ON BRIEF

Before WILLIAM F. SMITH, GRIMES, and GREEN, Administrative Patent Judges.

GREEN, Administrative Patent Judge.

DECISION ON APPEAL

This is a decision on appeal under 35 U.S.C. § 134 from the examiner's final rejection of claims 36, 51 and 52. The claims read as follows:

36. A monoclonal antibody that selectively binds to a 16 ± 4 kDa antigen of Sarcocystis neurona wherein the antigen is isolated from Sarcocystis neurona merozoites by two-dimensional polyacrylamide gel electrophoresis and used to produce a hybridoma which produces the monoclonal antibody.

51. A monoclonal antibody that selectively binds to a 30 ± 4 kDa antigen of Sarcocystis neurona wherein the antigen is isolated from Sarcocystis neurona merozoites by two-dimensional

Docketed
Sept 29, 2005

c: J. Sherman
8/3/05

polyacrylamide gel electrophoresis and used to produce a hybridoma which produces the monoclonal antibody.

52. A monoclonal mixture comprising an antibody that selectively binds to a 16 ± 4 kDa antigen of Sarcocystis neurona and a monoclonal antibody that selectively binds to a 30 ± 4 kDa antigen of Sarcocystis neurona wherein the antigens are separately isolated from Sarcocystis neurona merozoites by two-dimensional polyacrylamide gel electrophoresis and separately used to produce hybridomas which produce the monoclonal antibodies for the mixture.

The examiner relies upon the following references:

Liang et al. (Liang 1997), "Micropreparative High Resolution Purification of Proteins by a Combination of Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis, Isoelectric Focusing, and Membrane Blotting," Anal. Biochem., Vol. 250, pp. 61-65 (1997).

Liang et al. (Liang 1998), "Evidence that Surface Proteins Sn14 and Sn16 of *Sarcocystis neurona* Merozoites Are Involved in Infection and Immunity," Infection and Immunity, Vol. 66, No. 5, pp. 1834-1838 (1998)

Harlow et al. (Harlow), Antibodies, A Laboratory Manual, Chapter 6, Cold Spring Harbor Press (1988).

Claims 36, 51 and 52 stand rejected under 35 U.S.C. § 103(a) as being unpatentable over the combination of Liang 1998 or Liang 1997 and Harlow. After careful review of the record and consideration of the issue before us, we affirm.

DISCUSSION

The claims stand rejected under 35 U.S.C. § 103(a) as being obvious over the combination of Liang 1998 or Liang 1997 and Harlow.

Liang 1998 is cited for teaching the identification of S. neurona merozoite antigens from samples from horses with neurological signs typical of equine

myeloencephalitis (EPM) or confirmed EPM. See Examiner's Answer, page 3. The antigens so identified include a 30 KD antigen and a 16 KD antigen, which, according to the reference, appear to be cell surface antigens of merozoites. See id. The examiner states that "[a] combination of the results of western-blot analysis (figure 1) and trypsin digestion (figure 3 B) suggests that these are important surface proteins that could be used in specific diagnosis of S. neurona infection, as candidate antigens for vaccine development and specific antibodies to these antigens lyse merozoites via complement or inhibit their attachment and penetration to host cells." Id. Liang 1998 is also cited for teaching that monoclonal antibodies are often used to study parasitic proteins. See id. The examiner acknowledges that while "[a]ntibodies to 16KD antigen not only recognized the 16KD antigen but also lysed the merozoites in in vitro neutralization assays . . . antibodies to 30KD recognized the 30KD antigen but could not inhibit in vitro neutralization of merozoites, as 30KD antigen appears to cross-react with serum obtained from horses infected with other Sarcocystis species." Id. at 4. The examiner concludes "[t]hus the prior art teaches 30KD, 16KD, 14KD and 11KD proteins as merozoite surface antigens that are involved in S. neurona infection and EPM and could be used in the specific diagnosis of S. neurona." Id.

Liang 1997 is cited for teaching "purified 30 KD and 19 KD (i.e. 16 KD +/- 4) antigens from S. neurona merozoites by using infected horses serum." Id.

The rejection acknowledges that "Liang 1998 or Liang 1997 does not teach monoclonal antibody that selectively binds to 16KD antigen or a monoclonal antibody that selectively binds to a 30KD antigen or a monoclonal antibody mixture." Id.

Harlow is cited for teaching methods for "making monoclonal antibodies to any given antigen." Id.

The rejection concludes:

> It would have been prima facie obvious to one, having ordinary skill in the art at the time the invention was made to make monoclonal antibodies to merozoite surface antigens including 16KD and 30KD because Liang [ ] taught detection of S. Neurona infection using serum and sometimes infected horse serum cross reacts with antigens such as 30KD. Further, the art suggests monoclonal antibodies are often used to study parasite (page 1837, last paragraph) proteins and EPM disease occurs after merozoite passes through the vascular endothelium of blood-brain barrier into the central nervous system, and so humoral responses play essential role in blocking this migration and specific cytotoxic T cells are ineffective in attacking merozoite migration to the central nervous system in the blood stream (pag[e] [sic] 1837, left column, third paragraph). Therefore an artisan of ordinary skill would have been motivated to use readily available and purified surface antigens (Liang [ ] 1997) from merozoites including 16KD and 30 KD as disclosed by the prior art Liang [ ] 1998 or Liang [ ] 1997 with a reasonable expectation of success for raising monoclonal antibodies by using well established hybridoma technology as taught by [Harlow] because Liang [ ] 1998 suggests that humoral immunity to S. neurona infection is important (see page 1836 under discussion) especially in EPM disease and surface antigens including 30KD, 16KD, 14KD are immunoreactive with infected serum that are useful for the detection of the pathogenic S. neurona and Liang [ ] 1997 teach purification of target merozoite proteins 19 KD, 30 KD and 100KD. Moreover, it has become routine in the art to make monoclonal antibodies for characterizing and purifying proteins especially target proteins such as surface proteins of parasites or envelope proteins of bacteria.

Id. at 4-5.

We initially note that we find the Liang 1997 reference to be cumulative to the Liang 1998 reference, thus we focus our analysis on the Liang 1998 reference.

It is axiomatic that "the Examiner bears the burden of establishing a prima facie case of obviousness based upon the prior art. '[The Examiner] can satisfy this burden only by showing some objective teaching in the prior art or that knowledge generally available to one of ordinary skill in the art would lead that individual to combine the relevant teachings of the references.'" In re Fritch, 972 F.2d 1260, 1265, 23 USPQ2d 1780, 1783 (Fed. Cir. 1992) (citation omitted). An adequate showing of motivation to combine requires "evidence that 'a skilled artisan, confronted with the same problems as the inventor and with no knowledge of the claimed invention, would select the elements from the cited prior art references for combination in the manner claimed.'" Ecolochem, Inc. v. Southern Calif. Edison Co., 227 F.3d 1361, 1375, 56 USPQ2d 1065, 1076 (Fed. Cir. 2000). We find that the rejection establishes a prima facie case of obviousness that has not been rebutted by appellants, and the rejection is affirmed.

Appellants argue that the combination does not provide any motivation to make the claimed monoclonal antibodies or the claimed mixture of antibodies. See Appeal Brief, page 6. Appellants acknowledge that "[g]enerating antibodies to a given antigen or epitope may have become routine in recent years," id. at 7,

but contend that "[t]here must also be a clear objective or motivation for one skilled in the art to combine the prior art." See id. Appellants assert that "the prior art references do not identify any need for making antibodies against the [16 and 30] kDa antigens or identify any problem that the antibodies could be used to solve." Id. at 14. Appellants contend that the rejection apparently relied on the "notion that making antibodies is routine" to provide the motivation to combine Liang 1998 with Harlow, which, according to appellants, is an impermissible hindsight rejection. See id. at 14.

Appellants argue with respect to claims 51 and 52 that the ordinary artisan would not have had motivation to make antigens against the 30 kDa antigen, because, while Liang 1998 suggests that the 16 kDa antigen appears to be an important antigen, Liang 1998 "suggests that the 30 kDa antigen is not important because it had no inhibitory activity and antibodies against the antigen were not recognized to be specific." Appeal Brief, page 15 (references omitted). Thus, appellants conclude, the ordinary artisan would not have been motivated to produce monoclonal antibodies against the 30 kDa antigen as in claim 51, or to produce a mixture containing that antibody as in claim 52.

Appellants' arguments are not convincing. As noted by the rejection, Liang 1998 specifically teaches that "monoclonal antibodies are often used to study parasitic proteins." See Liang 1998, page 1837, Col. 2. Moreover, Liang 1998 also teaches that *Sarcocystis neurona* is the etiologic agent of equine protozoal myeloencephilitis, see Liang 1998, page 1834, Abstract and Col. 1,

thus providing motivation to study and detect the parasite. Liang 1998 also teaches that antibodies specific for the 16 kDa antigen have protective activity against *S. neurona* and "support the use of the immunoblot test in diagnosis of [equine protozoal myeloencephilitis]," thus providing motivation to generate antibodies against the 16 kDa antigen.

With respect to the 30 kDa antigen, although Liang 1998 teaches that the antibodies are immunoreactive with sera from horses, infected with other *Sarcocystis*, it also teaches that monoclonal antibodies are often used to study parasitic proteins. Therefore, one of ordinary skill would have been motivated to produce antibodies to the 30 kDa antigen in order to study the antigen in *S. neurona* and other *Sarcocystis*. And one of ordinary skill would understand that a mixture of monoclonal antibodies to the 16 and 30 kDa antigens would allow one to determine if *Sarcocystis* other than *S. neurona* were present in horses with equine protozoal myeloencephilitis.

Appellants argue further that the combination of Liang 1998 and Harlow does not enable one skilled in the art to prepare antibodies against the 16 and 30 kDa antigens. See Appeal Brief. While, according to appellants, Liang 1998 demonstrates that the antigens are antigenic in horses, Harlow relates to the production of monoclonal antibodies in mice, and there is no teaching or suggestion in Liang 1998 that the antigens would produce antibodies, i.e., be antigenic, in mice. See id. at 11-12. Appellants distinguish Ex parte Erlich, 3 USPQ2d 1001 (Bd. Pat. App. & Int. 1986) by arguing that "because even though

Liang . . . (1998) show[s] that the 16 and 30 kDa antigens are antigenic, there is no prior art which shows that the method of [Harlow] could be adapted to produce monoclonal antibodies against the [16 and 30] kDa antigens." Id. at 13-14. Appellants conclude that the prior art does not provide a reasonable expectation of success that monoclonal antibodies could be generated against the 16 and 30 kDa antigens. See id. at 14.

Again, appellants' arguments are not found to be convincing. As acknowledged by appellants, methods for obtaining and screening for monoclonal antibodies were well known at the time of invention. See also In re Wands, 858 F.2d 731, 736, 8 USPQ2d 1400, 1404 (Fed. Cir. 1988). Because the 16 and 30 kDa antigens from *S. neurona* were antigenic in horses, one of ordinary skill in the art would expect them to be antigenic in other mammals, such as mice. Moreover, appellants themselves refer to the 16 and 30 kDa proteins from *S. neurona* as antigens, and define an antigen as "a substance which stimulates production of antibody or sensitized cells during an immune response," Specification, page 12, thus one of ordinary skill would expect the 16 and 30 kDa antigens from *S. neurona* to be antigenic in mice. Finally, all that is required is a reasonable expectation of success, not absolute predictability of success. See In re O'Farrell, 853 F.2d 894, 903, 7 USPQ2d 1673, 1681 (Fed. Cir. 1988).

CONCLUSION

Because the examiner has established a prima facie case of obviousness that has not been rebutted by appellants, the rejection of claims 36, 51 and 52 under 35 U.S.C. § 103(a) is affirmed.

No time period for taking any subsequent action in connection with this appeal may be extended under 37 CFR § 1.136(a).

AFFIRMED

William F. Smith
Administrative Patent Judge

Eric Grimes
Administrative Patent Judge

Lora M. Green
Administrative Patent Judge

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